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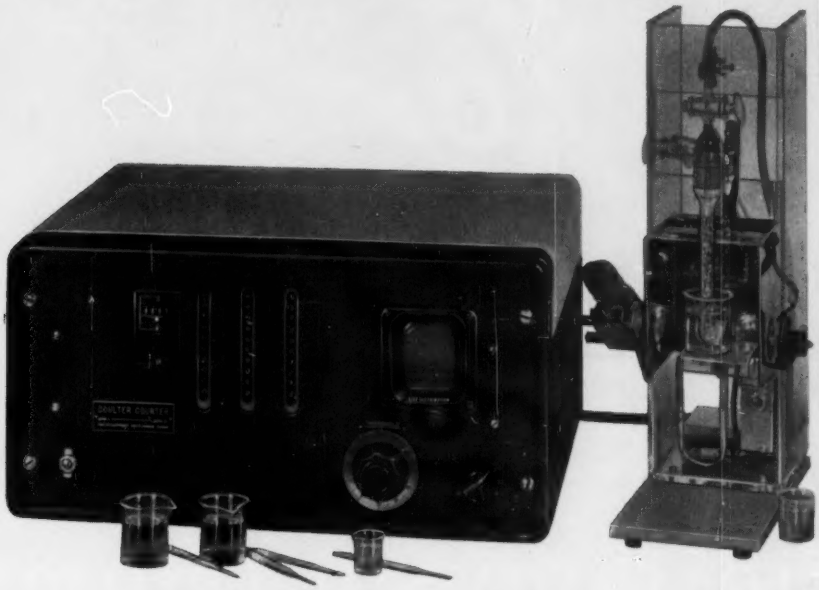

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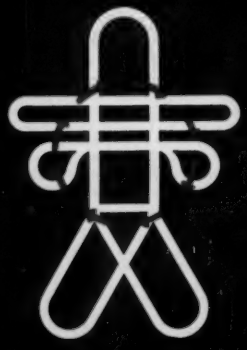

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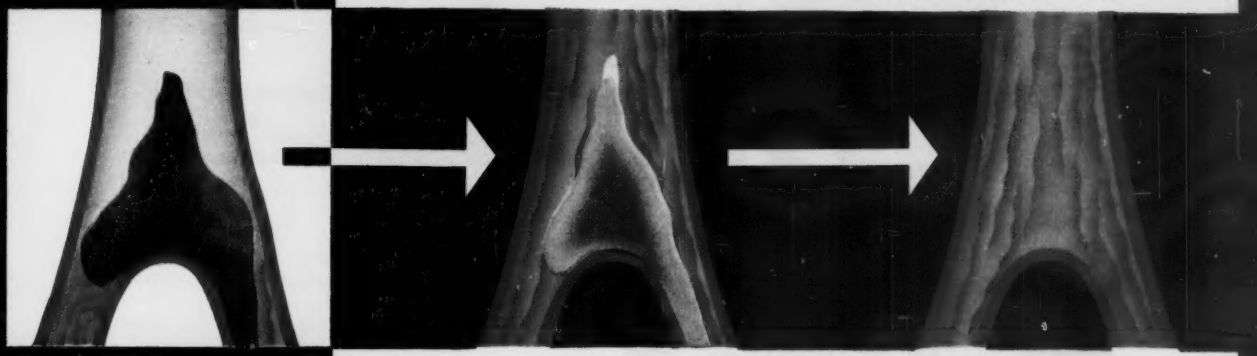

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Cirrhose et Cancer Primitif du Foie. R. FAUVERT et J.-P. BENHAMOU.

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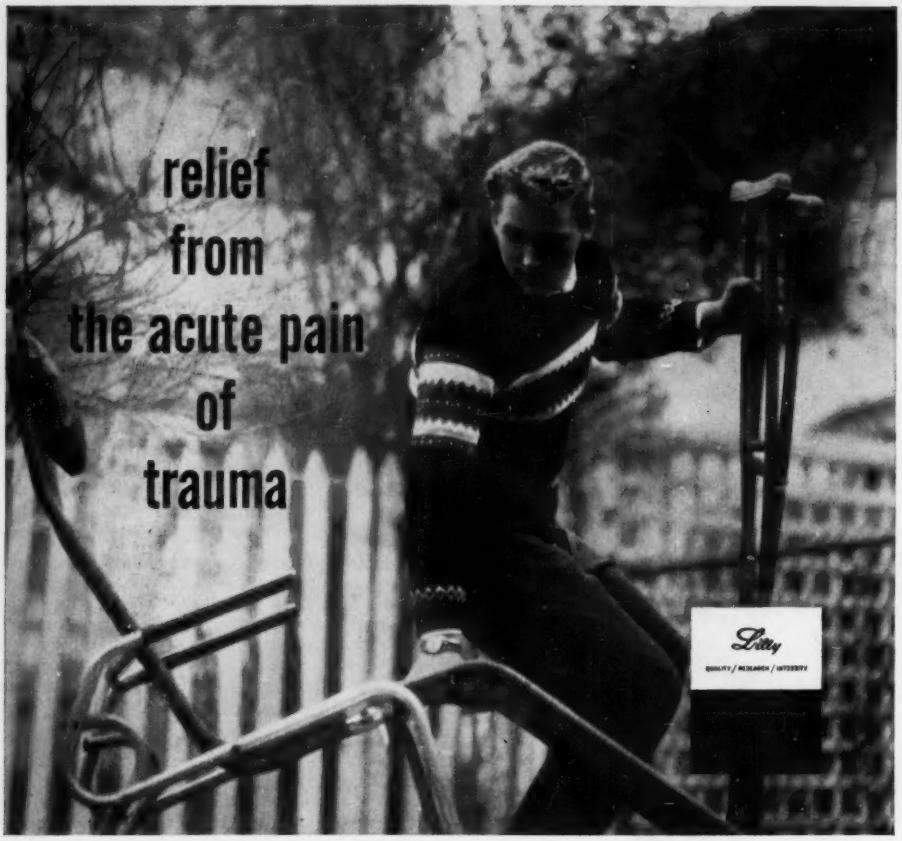

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ANTITHYROID ACTIVITY ELICITED BY THE INGESTION OF PURE PROGOITRIN, A NATURALLY OCCURRING THIOGLYCOSIDE OF THE TURNIP FAMILY *

By MONTE A. GREER AND J. M. DEENEY

(From the Division of Endocrinology, Department of Medicine, University of Oregon Medical School, Portland, Ore.)

(Submitted for publication March 17, 1959, accepted April 2, 1959)

Foodstuffs of various sorts have been implicated as a cause of goiter for many years. Only by the discovery of Chesney, Clawson and Webster in 1928 that cabbage feeding would result in thyroid enlargement in laboratory animals (1) were these vague hypotheses started on a firm experimental footing, however (for detailed review see Reference 2).

Although various articles of diet have been reported to produce goiter in diverse animal species since that time, studies employing the acute inhibition of thyroidal radioiodine accumulation indicated that only a relatively small number of foods possessed antithyroid activity in man (3). Of these, rutabaga was consistently the most potent. Eventually it was possible to isolate and identify the active agent in this species, *l*-5-vinyl, 2-thiooxazolidone (4), more recently termed "goitrin" (5). Goitrin has been identified in the seeds of most Brassicae but until recently in the edible parts of only rutabaga and turnip. It has now been reported that goitrin has been detected in the edible portions of cabbage, kale and rape (6, 7). As yet no other naturally occurring antithyroid compound with pathogenetic significance for man is known to exist.

Very early in the studies isolating goitrin it became apparent that this substance did not exist in the free state but was bound to a precursor from which it could be liberated by a crude enzyme preparation, myrosin, prepared from crucifers. Myrosin supposedly is or contains a specific thioglycosidase which hydrolyzes mustard oil thioglycosides (8).

The original studies of the antithyroid activity of foods in man had indicated that cooking would usually destroy the potency of rutabaga and turnip. Goitrin also could not be isolated from cooked material but would appear when such preparations were treated with myrosin (9). This led to the assumption that the precursor, called "progoitrin," could be hydrolyzed only by the myrosin contained in the crucifer and that cooking, by denaturing the enzyme, would prevent the active goitrin from being formed. Thus only the ingestion of raw rutabagas or turnips was considered as potentially leading to the development of goiter (9).

Recently the isolation and identification of progoitrin as a thioglycoside has been accomplished (5). A relatively simple method of preparing sizable amounts of pure, crystalline progoitrin by elution from an anion exchange resin has been devised. This has permitted an experimental examination of our original hypothesis. To our surprise, we have found that progoitrin can be converted quantitatively to goitrin in the body by both man and rats without the addition of exogenous myrosin.

MATERIALS AND METHODS

Crystalline progoitrin was prepared from a 75 per cent acetone extract of ground rutabaga or kale seed by elution with sodium chloride from an anion exchange column. The method is to be presented in full in a separate paper (10). Purified goitrin and myrosin (8) were also prepared from rutabaga seed.

Goitrin was assayed in biological fluids by extracting with peroxide-free ether, evaporating the ether extract, dissolving the residue in distilled water and reading the absorption at 230, 240 and 250 mμ in a Beckman spectrophotometer. Goitrin has a maximum specific absorption at 240 mμ (4) as compared to 227 for progoitrin (5). In addition to the different absorption maxima they are easily distinguished by the fact that goitrin is equally soluble in ether and water while progoitrin is insoluble in ether.

* Supported in part by United States Public Health Service Grants A-2503 and A-1447. Presented in part at the 1958 Meeting of the Western Society for Clinical Research. Greer, M. A., and Deeney, J. M. Physiologic studies with pure crystalline progoitrin. Clin. Res. 1958, 6, 90.

Acute tests of antithyroid activity in man were made by two methods. In one a modification of the technique of Stanley and Astwood (11), utilizing a change in the slope of the "accumulation gradient," was employed. Ten μc. of I^{131} was administered to subjects and counts made over the thyroid region with a collimated scintillation counter every 10 to 20 minutes for the first four hours and every 45 to 60 minutes for the next four hours. A final reading was taken at 24 hours. Counts taken over the lower thigh were assumed to represent background and were subtracted from the neck radioactivity at each interval. Each corrected neck count was plotted as per cent of the administered dose on the ordinate against the square root of the time in minutes from administration of the radioiodine on the abscissa. From such a plot a straight line is formed which persists for the first eight to 12 hours. After the slope of the accumulation gradient had been determined, progoitrin with or without added myrosin was administered orally. Antithyroid activity could be detected by a deviation of the subsequent neck counts below the extrapolated slope. In addition, the expected 24 hour uptake, as calculated by a previously determined formula using the slope of the accumulation gradient (12) was compared with that actually obtained.

The second method employed an initial 24 hour thyroidal radioiodine uptake. Progoitrin was then administered orally and followed by a second 24 hour uptake study one to three hours later. Urine was collected quantitatively and assayed for goitrin by the method described above. Blood was also drawn for goitrin assay at various intervals before and after administration of progoitrin.

In two subjects, purified but noncrystalline goitrin was given to compare its metabolism and activity with progoitrin.

In three patients, progoitrin was administered through a 30 cm. rectal tube. The patients were placed in a prone position with the trunk flexed downward almost 90° over the edge of the table. Progoitrin was dissolved in 20 ml. of saline and allowed to flow into the colon by gravity. Two successive rinsings, each with 20 ml. saline, were then made through the tube.

In one patient with untreated recurrent Graves' disease, a single dose of 1 Gm. progoitrin was given once daily over a period of one month to evaluate its efficacy in the treatment of thyrotoxicosis when administered in this manner.

The antithyroid potency of progoitrin was also tested in 100 to 120 Gm. male Holtzman rats. It was given orally, subcutaneously or intraperitoneally in physiologic saline at various intervals prior to administration of 0.2 μc. I^{131} in 0.2 ml. physiologic saline intraperitoneally. The animals were killed four and one-half hours after receiving radioiodine, the thyroids dissected and their radioactivity determined in a well scintillation counter.

RESULTS

I. *Studies in man*

A. Acute inhibition of radioiodine uptake. The inhibition of the "accumulation gradient" following the ingestion of 0.5 to 2.0 Gm. of progoitrin orally was studied in one thyrotoxic and seven euthyroid volunteers. The results are summarized in Table I and in Figures 1 and 2. In five of these subjects, myrosin was given in addition at the same time; in two of these five the progoitrin had been incubated with myrosin overnight at 37° in a pH 5.6 phosphate buffer. The degree of inhibition of the accumulation gradient was arbitrarily graded from zero to five according to the system previously used (3).

It is apparent that inhibition of the acute uptake of radioiodine was produced by the ingestion of progoitrin. Prior incubation of progoitrin with myrosin or the addition of this enzyme at the time of progoitrin administration did not seem to affect the results materially.

The interval from ingestion of progoitrin to a

TABLE I

Inhibition of accumulation gradient by oral progoitrin (PG) in euthyroid patients *

Patient	Accumulation gradient	Interval	Dose PG	Myrosin	Expected 24 hr. uptake	Actual 24 hr. uptake	Grade of inhibition†
		min.	*Gm.*				
W. H.	1.28	216	0.5	+‡	34.6	24.5	2
M. K.	1.28	139	0.5	+	34.6	26.6	2
C. K.	1.95	95	2.0	+	48.4	22.5	5
M. M.	1.20	166	2.0	+	32.7	16.1	5
A. K.	1.37	116	2.0	+‡	36.6	18.8	5
M. L.	0.58	251	2.0		17.0	10.5	5
L. R.	0.68	104	2.0		19.7	12.0	5

* Interval = time from administration of progoitrin to beginning inhibition of radioiodine uptake. Ten ml. myrosin was given with progoitrin where indicated.

† Grade of inhibition is based on a scale of 0 = no effect and 5 = complete inhibition for 24 hours (3).

‡ Myrosin was incubated with progoitrin for 18 hours before administration.

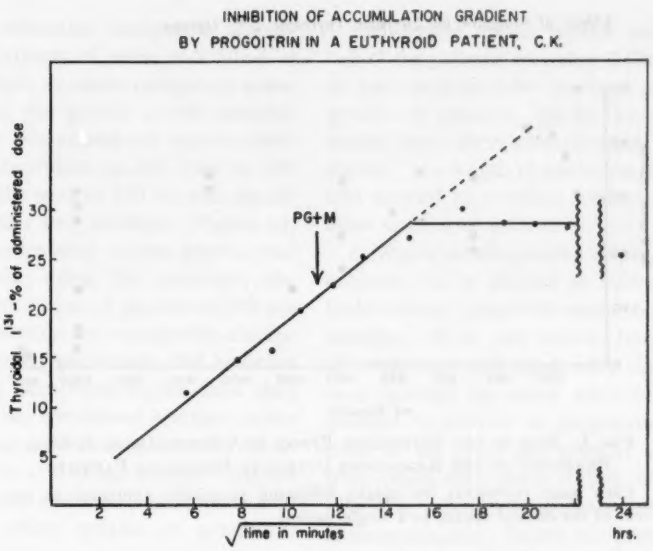

FIG. 1. INHIBITION OF ACCUMULATION GRADIENT BY SIMULTANEOUS ORAL ADMINISTRATION OF 2 GM. PROGOITRIN AND 10 ML. MYROSIN SOLUTION (PG + M)

detectable change in the slope of the accumulation gradient was somewhat longer than had previously been obtained when raw rutabagas and turnips were fed.

Inhibition of radioiodine uptake by progoitrin appeared to be just as effective in the thyrotoxic as in the euthyroid patients. The day prior to the progoitrin study, the 24 hour radioiodine uptake in this patient was 71 per cent. Following the appearance of obvious inhibition of the accumula-

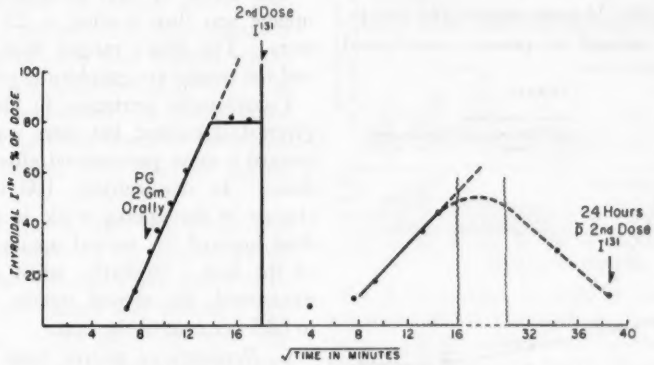

FIG. 2. INHIBITION OF ACCUMULATION GRADIENT IN A THYROTOXIC PATIENT (L.B.) GIVEN 2 GM. PROGOITRIN ORALLY

The "second dose" of I^{131} on the graph is referred to as the third dose of I^{131} in the text since the patient had an initial radioiodine uptake before the studies graphically presented here were undertaken. The radioactivity remaining from the preceding dose of radioiodine was subtracted from the thyroidal counts in calculating the per cent of the administered dose in the thyroid.

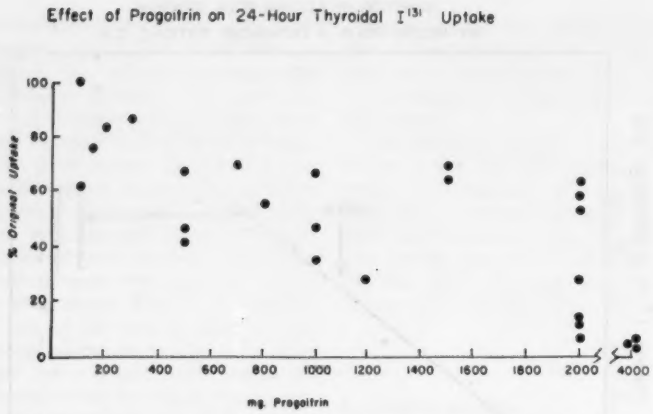

FIG. 3. PLOT OF THE SUPPRESSIVE EFFECT OF VARYING DOSES OF ORAL PROGOITRIN ON THE RADIOIODINE UPTAKE IN EUTHYROID PATIENTS

Each point represents the uptake following progoitrin expressed as per cent of the control uptake in a single patient.

tion gradient by progoitrin, a third tracer dose of radioiodine was given. Although a considerable portion of this third dose rapidly accumulated in the thyroid gland, most of it had been lost from the thyroid 24 hours later (Figure 2). The 24 hour thyroidal accumulation of the third dose was only 11 per cent compared to the previous 71 per cent. The rapid concentration and release of this last tracer dose is characteristic of the behavior one would expect of unbound iodide.

B. Inhibition of the 24 hour radioiodine uptake. Since progoitrin seemed to possess antithyroid activity when administered without added myrosin, in the following studies progoitrin alone was used. As a preliminary control study (although the technique had previously been investigated) two consecutive 24 hour uptakes were performed in seven euthyroid subjects. The mean value for the second 24 hour uptake was 111 ±1.9 per cent of the initial uptake. A second uptake lower than the first was not observed.

The effect of oral progoitrin on the second uptake was then studied in 23 additional volunteers. The doses ranged from 0.1 to 4.0 Gm. and the results are graphically plotted in Figure 3.

Considerable variation in the response to a given dose existed, but there was a definite trend toward a more pronounced effect with increasing doses. In one subject, 100 mg. produced no change in the uptake while in another the same dose lowered the second uptake to 61.5 per cent of the first. Similarly, when 2.0 Gm. was administered, the second uptake ranged from 6.0 to 62.5 per cent of the first.

C. Recovery of goitrin from blood and urine. Complete aliquot collections of urine and serial samples of blood extending over a period of several days were studied in nine subjects following ingestion of 150 mg. to 4.0 Gm. progoitrin. Only in those receiving at least 1.5 Gm. progoitrin was it possible to clearly demonstrate goitrin in the blood and urine.

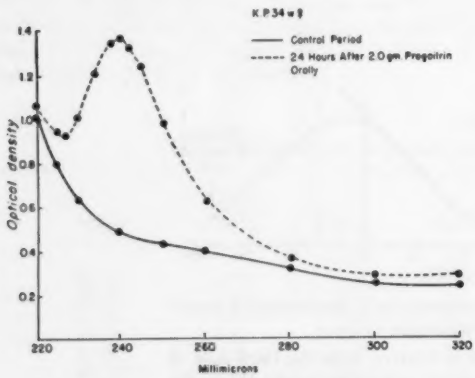

FIG. 4. ETHER EXTRACTS OF URINE

Note the absorption peak at 240 mμ characteristic of goitrin which appears in ether extracts of urine after administration of progoitrin.

Because of the inconstant nonspecific absorption of the ether extracts of urine and blood at 240 mμ, it was difficult to obtain completely quantitative estimates of the goitrin in the samples. A positive response was considered one in which there was higher absorption at 240 than at 230 or 250 mμ. When the peak at 240 mμ was significantly above the other two readings (Figure 4), a semiquantitative calculation of the goitrin concentration was made, using the previously determined specific absorption of goitrin at 240 mμ (4). Since no correction for nonspecific absorption was made in these calculations, the recorded values are probably somewhat higher than they should be. It was not considered practical to try to devise an adequate correction system, however. A peak of absorption at 240 mμ in ether extracts of urine and blood was never seen in patients who had not received either goitrin or progoitrin (Figure 4).

When 4 Gm. of progoitrin was administered orally, goitrin was detected in both blood and urine for up to four days. In one patient studied one month after ingestion of 10 mMoles (4 Gm.) of progoitrin, 10 mMoles of goitrin (1.29 Gm.) was given and similar blood and urine studies made (Figure 5). The total excretion of goitrin in both instances was almost identical (103 vs. 108 mg. over the entire period). However, after progoitrin administration the serum goitrin concentration reached a maximum at approximately 35 hours, while a maximum was reached within the first half hour after goitrin was given. The excretion and blood concentration of goitrin in the other patients receiving large doses of progoitrin and in one other patient who received goitrin were similar.

No ill effects were seen after oral progoitrin, but both patients who received the purified goitrin became nauseated and vomited a few hours later. In one of the patients, nausea and vomiting persisted for two days. Since gastrointestinal distress had never been seen in the earlier studies with pure crystalline goitrin (9), it is unlikely that this discomfort was produced by the goitrin *per se*. A more reasonable explanation is that the purified goitrin preparation used in the present studies contained trace amounts of offensive impurities responsible for the nausea. The material had an unpleasant aromatic taste and odor.

Radioiodine uptake studies were performed in two of the patients receiving 4 Gm. progoitrin and in both patients who received goitrin. In both groups of patients, uptake was suppressed for several days after administration of the test substance. The length of inhibition of radioiodine uptake seemed to correlate well with the blood and urine content of goitrin.

D. Rectal administration of progoitrin. Certain evidence, to be alluded to below, indicated that hydrolysis of progoitrin might occur in the large intestine. It is well known, however, that some substances are not appreciably absorbed once they pass through the small intestine. To ascertain whether hydrolysis of progoitrin might actually occur in the colon and whether absorption therefrom could take place, 4 Gm. of progoitrin dissolved in saline was administered by rectal tube to three subjects. In two of the volunteers, goitrin could subsequently be detected in the urine and in one of these it was also found in the blood. One

URINARY EXCRETION AND PLASMA CONCENTRATION OF GOITRIN AFTER ORAL OR RECTAL ADMINISTRATION OF GOITRIN OR PROGOITRIN

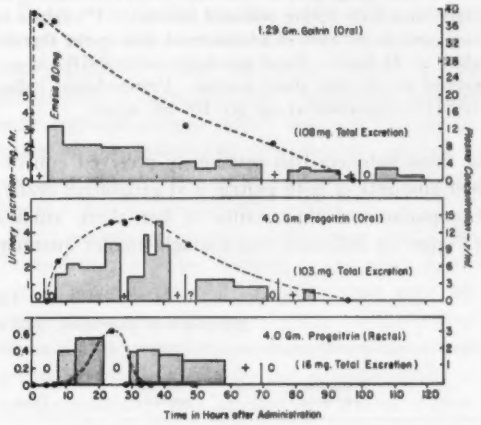

FIG. 5. SUCCESSIVE STUDIES OF SERUM CONCENTRATION AND URINARY EXCRETION OF GOITRIN IN THE SAME PATIENT (M.M.) AFTER ADMINISTRATION OF EQUIVALENT AMOUNTS OF ORAL AND RECTAL PROGOITRIN AND ORAL GOITRIN

Note emesis of 8.0 mg. goitrin following oral goitrin administration. The dotted line represents serum concentration and the solid bars urinary excretion. 0 = no goitrin detectable; + = goitrin detectable but urine volume unknown or amount of goitrin too small to make accurate calculation; ? = urine sample inadvertently discarded.

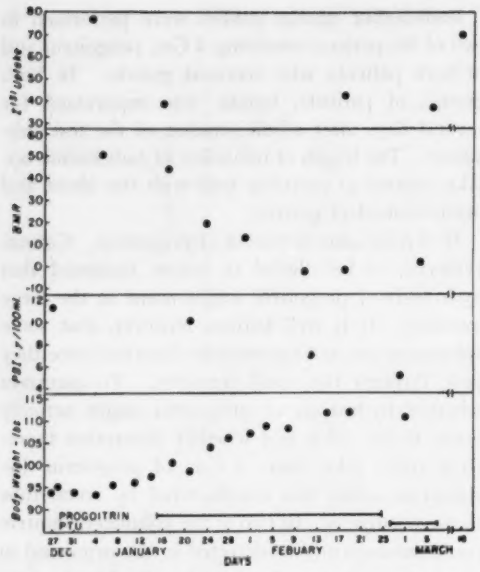

FIG. 6. GRAVES' DISEASE TREATED WITH 1 GM. PROGOITRIN OR 300 MG. PROPYLTHIOURACIL (PTU) ONCE DAILY (PATIENT W.L.)

One Gm. progoitrin or 300 mg. propylthiouracil was given once daily during indicated intervals. I^{131} uptake is expressed as per cent of administered dose in the thyroid gland at 24 hours. Basal metabolic rate (BMR) is expressed as per cent above normal. Protein-bound iodine (PBI) is expressed as μg. per 100 ml. serum.

of these subjects had previously received equivalent amounts of both goitrin and progoitrin orally. Comparison of the results of her three studies (Figure 5) indicated that a much smaller quantity of goitrin was excreted in the urine after rectal than after oral administration (16 vs. 103 mg).

The one patient in whom goitrin could not be detected developed a watery diarrhea soon after instillation of the progoitrin. It is possible that the failure to detect the liberation of goitrin in this case was due to elimination of the progoitrin before adequate hydrolysis had occurred. Unfortunately, it was not possible to obtain stool analyses in any of the three subjects.

E. Therapy of hyperthyroidism. In order to assess the clinical efficacy of the apparent antithyroid effect of progoitrin, 1 Gm. once daily in a glass of orange juice was administratered to a patient with fairly severe recurrent Graves' disease. Serial determinations were made of the patient's weight, serum protein-bound iodine, radioiodine uptake and basal metabolic rate during the month of progoitrin therapy. In addition, the effect of a comparable dose of propylthiouracil (300 mg.) once daily was studied for a period of nine days beginning at the conclusion of the progoitrin period.

The results are summarized in Figure 6. It can be seen that a gratifying clinical response to progoitrin was made. There was a progressive fall in the BMR and PBI and a gradual increase in body weight beginning very shortly after initiation of progoitrin therapy. The 24 hour radioiodine uptake was decreased from a pretreatment level of 77 per cent to 39 to 44 per cent during the progoitrin period. A similar value of 39 per cent was obtained when medication had been shifted

TABLE II

Effect of oral progoitrin, with or without myrosin, on I^{131} uptake *

Experiment and group	No. of animals	Dose	I^{131} uptake	
			Dose	Controls
		mg.	%	%
I				
Control	4		1.25 ± 0.03	100.0 ± 2.4
PG	4	20	1.04 ± 0.21	83.2 ± 16.8
PG + M	4	20	2.31 ± 0.37	184.8 ± 29.6
PTU	4	10	0.10 ± 0.01	8.0 ± 0.8
II				
Control	6		1.37 ± 0.34	100.0 ± 24.8
PG	6	200	0.91 ± 0.28	66.4 ± 20.4
PG + M	6	200	0.17 ± 0.04	12.4 ± 3.1
PTU	6	5	0.21 ± 0.12	15.3 ± 8.8

* Results are expressed as mean and standard error. Animals were maintained on Purina Laboratory Chow®. PG = progoitrin; M = 1 ml. myrosin given orally simultaneously with progoitrin; PTU = propylthiouracil, administered subcutaneously.

TABLE III

Inhibition of I^{131} uptake by progoitrin when given at varying intervals preceding radioiodine *

Experiment and group	No. of animals	Dose	Hours preceding I^{131}	I^{131} uptake	
				Dose	Controls
		mg.		%	%
III					
Control	5			3.11 + 0.40	100.0 ± 12.9
PG + M	5	100	19	1.28 ± 0.26	41.0 ± 8.4
PG + M	5	100	1	0.83 ± 0.13	26.7 ± 4.2
PTU	5	10	1	0.23 ± 0.03	7.4 ± 1.0
IV					
Control	5			3.78 ± 0.36	100.0 ± 9.5
PG	5	300	23.5	1.62 ± 0.21	42.9 ± 5.6
PG	5	300	19.5	1.92 ± 0.42	50.8 ± 11.1
PG	5	300	17.5	1.56 ± 0.27	41.3 ± 7.1
PG + M	5	300	17.5	1.35 ± 0.37	35.7 ± 9.8
PG	5	300	1	1.43 ± 0.31	37.8 ± 8.2
PG + M	5	300	1	0.96 ± 0.22	25.4 ± 5.8
PTU	5	10	1	0.37 ± 0.12	9.8 ± 3.2

* Progoitrin was given orally at indicated intervals preceding I^{131}. Abbreviations are identical to those in Table II. Rats were fed Purina Laboratory Chow®.

to propylthiouracil. Eleven days after propylthiouracil had been discontinued, the uptake had risen again to 70 per cent.

II. *Studies in the rat*

A. Oral progoitrin. Progoitrin, with or without myrosin, was given by stomach tube in several experiments (Table II). Inhibition of radioiodine uptake was regularly induced when an adequate dose of progoitrin was administered, but inhibition did not equal that obtained from the injection of 5 to 10 mg. propylthiouracil. Previous studies had shown that propylthiouracil was 55 times more active than goitrin in the rat (9). Since enzymatic hydrolysis of progoitrin will yield only 32.2 per cent goitrin by weight, 1.7 Gm. of progoitrin (assuming complete hydrolysis) would be required to equal 10 mg. propylthiouracil. Unfortunately, when large doses were given the animals developed diarrhea and became ill. Thus the failure to obtain a suppression from progoitrin equivalent to that produced by propylthiouracil may be attributed to the rapid passage of the orally administered material through the intestinal tract before adequate hydrolysis or absorption could take place.

Because of the relatively slow hydrolysis of progoitrin, we were not sure what interval between progoitrin and I^{131} administration would be optimal to detect antithyroid activity. For this reason the time interval between administration of the two materials was varied in some experiments (Table III). No significant dissimilarity was seen, although a shorter interval appeared to produce somewhat greater depression of the I^{131} uptake.

During the early studies with orally administered progoitrin, the rats were fed Purina Laboratory Chow®. Since the iodine content of this preparation is quite high, the radioiodine uptakes were correspondingly rather low and the significance of the differences between the groups is probably not as great as in the later experiments where a low iodine diet was used.

B. Comparison of oral vs. systemic progoitrin. To ascertain whether hydrolysis occurred in the gut or after unchanged progoitrin had been absorbed from the alimentary tract, comparison of the inhibitory effect of the same amounts of progoitrin administered orally and subcutaneously or intraperitoneally was studied in several experiments (Table IV). With lower doses of progoitrin, a much more pronounced inhibition of radioiodine uptake was produced with oral than with systemic administration. When larger doses were employed, however, there was some increase in the efficacy of systemic progoitrin whereas oral progoitrin became less effective. This decreased potency with increasing doses of oral progoitrin was probably related to the severe diarrhea which occurred when large doses were tube fed and has been mentioned above.

TABLE IV

Comparison of inhibition produced by oral vs. systemic progoitrin *

Experiment and group	No. of animals	Route of administration	Dose	Hours preceding I^{131}	I^{131} uptake	
					Dose	Controls
			mg.		%	%
V						
Control	6				38.81 ± 2.79	100.0 ± 7.2
PG	6	p. o.	200	3	13.11 ± 0.79	33.8 ± 2 0
PG	6	s. c.	200	3	31.28 ± 3.03	80.6 ± 7.8
PTU	6	s. c.	5	3	1.96 ± 0.28	5.1 ± 0.7
VI						
Control	6				37.80 ± 3.28	100.0 ± 8.7
PG	6	p. o.	625	3	12.03 ± 4.43	31.8 ± 11.7
PG	6	s. c.	625	3	27.90 ± 5.57	73.8 ± 14.7
PTU	6	s. c.	5	3	2.07 ± 0.22	5.5 ± 0.6
VII						
Control	4				26.62 ± 1.63	100.0 ± 6.1
PG	2	p. o.	1,000	17	22.01 ± 3.68	82.7 ± 13.8
PG	3	i. p.	1,000	17	14.93 ± 1.55	56.1 ± 5.8
PTU	3	s. c.	6	17	2.90 ± 1.94	10.9 ± 7.3
VIII						
Control	6				28.92 ± 1.32	100.0 ± 4.6
PG	6	p. o.	1,000	4	16.84 ± 1.11	58.2 ± 3.8
PG	6	i. p.	1,000	4	12.00 ± 3.28	41.5 ± 11.3
PTU	6	s. c.	5	2.5	1.65 ± 0.19	5.7 ± 0.7

* Progoitrin was given at indicated intervals preceding I^{131}. Abbreviations are identical to those in Table II; in addition, p. o. = orally, s. c. = subcutaneously, i. p. = intraperitoneally. All rats were fed a low iodine diet for one week prior to study.

DISCUSSION

It seems clear that our earlier hypothesis that progoitrin could only be converted to goitrin by exogenously supplied myrosin is in error. Although it is possible that progoitrin may possess some antithyroid activity itself, exclusive of the goitrin it produces, this does not seem likely. Other mustard oil thioglycosides with a structure similar to progoitrin have not been found to possess goitrogenic properties. Only those few thioglycoside aglycones which are converted to thionamides on hydrolysis are goitrogenic. Since goitrin is not present as such in the progoitrin molecule, but as an aliphatic secondary alcohol without a thionamide grouping and with cyclization to form goitrin occurring on hydrolysis (Figure 7), it is very unlikely that progoitrin by itself would have any activity. *In vitro* studies with thyroid slices in this laboratory have shown that goitrin will completely block organic binding of iodine whereas progoitrin is inactive (13). In addition to theoretical objections, from the studies made of the recovery of goitrin from blood and urine after administration of progoitrin, it appears that the precursor is quantitatively converted to the active antithyroid compound in the body. The calculated conversion would adequately explain the observed activity.

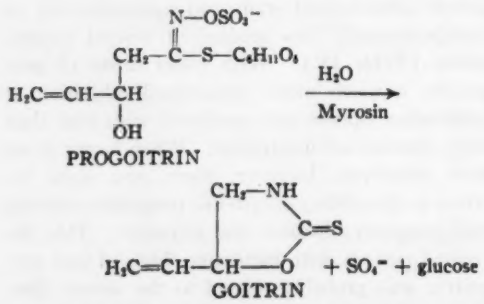

FIG. 7. ENZYMATIC CONVERSION OF PROGOITRIN TO GOITRIN BY MYROSIN

The site of hydrolysis is unknown at present. From the considerably less effective inhibition of radioiodine uptake produced by systemic compared to oral progoitrin it seems possible that this hydrolysis may occur predominantly in the gastrointestinal tract. The inhibition produced by large doses of systemic progoitrin may be due to escape of some of this material into the gut. *In vitro* studies of various tissues and body fluids

from rats and man have so far revealed thioglycosidase activity only in the gastrointestinal tract and its contents, including feces (13).

A number of attempts have been made to elucidate the origin of the thioglycosidase in feces. Since such activity had recently been reported in fungi (14), it appeared probable that some sort of microorganism was producing the fecal thioglycosidase. In spite of diligent effort, however, it has so far not been possible to isolate any thioglycosidase-producing organism from the stool (13). None of the common colon inhabitants seem to elaborate this enzyme.

Even if the thioglycosidase activity were due to gastrointestinal organisms, there was some question whether absorption of goitrin from the colon would occur. This was answered by the experiments in which progoitrin was administered by rectal tube. Significant quantities of goitrin were found to appear in the blood and urine following this procedure, although the quantities were much less than following oral administration of the same amount of progoitrin. However, in view of the appearance of goitrin in blood and urine and the concomitant demonstration of antithyroid activity as early as one to three hours after oral progoitrin administration, it is probable that some hydrolysis and absorption also occur higher in the gastrointestinal tract.

It is rather puzzling that consistent antithyroid activity was not detected in our earlier studies when cooked rutabagas and turnips were fed. In part this may be related to the delayed onset of inhibition of the accumulation gradient when pure progoitrin was given and the rather prompt onset of inhibition in the studies with whole foods. An effect from the cooked foods may have appeared so late as to be ignored as an artifact. Actually on re-examining the earlier data, it is apparent that occasionally an inhibition of the accumulation gradient did occur when cooked crucifers were given. Illustration of such inhibition was even published and commented on in the original article (3).

At present, we can say that, in most people, eating cooked vegetables containing progoitrin should be just as likely to produce goiter as eating them raw. There does seem to be some variation in the antithyroid effect of the same dose of progoitrin in different individuals (Figure 3) but it is not clear whether this is due to individual variations in the thioglycosidase activity of their gastrointestinal tracts or in the response of their thyroid glands to antithyroid compounds.

Progoitrin seems to hold some promise as an effective antithyroid compound in the treatment of thyrotoxicosis. Because of its prolonged effect when given in large doses, it was thought that the delayed hydrolysis of this compound would permit an adequate therapeutic effect if it were given only once daily or even less often. In the one hyperthyroid patient treated, a very satisfactory response was obtained over the month such therapy was exhibited.

It also appears that the prolonged effectiveness of single large doses of progoitrin is not entirely due to the slow hydrolysis after oral ingestion. When equivalent amounts of both goitrin and progoitrin were given to the same patient, although there was a much more rapid rise in blood levels after goitrin was given, the total length of time during which goitrin could be detected in blood and urine and the duration of radioiodine inhibition were the same with both drugs (Figure 5). The radioiodine uptake in the thyrotoxic patient under long-term treatment with progoitrin also was depressed to just as great an extent by an equivalent (300 mg.) dose of propylthiouracil administered once daily (Figure 6).

It is probable that the duration of action of progoitrin or goitrin is more related to the quantity given than to any peculiar metabolism of these compounds in comparison with other antithyroid substances. Such a direct relation of length of action to dosage had been demonstrated with a variety of goitrogenic drugs some time ago by Stanley and Astwood (11).

SUMMARY

Previous studies have shown that certain foods, especially rutabaga and turnip, possess antithyroid activity in man. The active antithyroid principle of the turnip family, goitrin, is released from a precursor, progoitrin, by enzymatic action. Earlier studies had indicated that cooking foods containing progoitrin would destroy myrosin, the specific hydrolytic enzyme which liberates goitrin from progoitrin. Therefore the cooked foods would have no antithyroid activity when eaten.

Studies were made with crystalline progoitrin in man and the laboratory rat employing inhibition of radioiodine uptake as an index of antithyroid activity. Contrary to expectations, it was found that progoitrin would produce consistent antithyroid activity either with or without simultaneous administration of myrosin. It was possible to identify the appearance of goitrin in the blood and urine by spectrophotometric analysis and to determine that the yield of goitrin from progoitrin was quantitative. This indicated that hydrolysis of progoitrin occurs within the body in the absence of exogenous myrosin. This demonstration of antithyroid activity following the administration of pure progoitrin suggests that our concept regarding the innocuousness of cooked crucifers in the production of simple goiter should be revised.

Progoitrin administered in a single daily dose of 1 Gm. was found an effective agent in the treatment of thyrotoxicosis in one patient.

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MEASUREMENT OF CHANGE IN TOTAL BODY FAT *

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(Submitted for publication February 10, 1959; accepted May 7, 1959)

Growing interest in the mechanisms and management of obesity has promoted investigation of methods for measuring total body fat, and has emphasized the need for accurate and practical methods of quantifying change in fat. Expressions based on either volume distribution or densitometric analysis lack validity when applied singly under conditions of abnormal hydration and provide doubtful accuracy in extreme obesity or severe emaciation (1). Substitution of parallel measurements of total body water and body density into the same fat prediction equation circumvents hydration abnormality, but requires the difficult measurement of body volume, which to date has been most satisfactorily accomplished by underwater weighing (2). More important, estimation of fat with methods that require densitometric analysis necessitates dependence upon the possibly invalid assumption of a known and constant density of the fat-free body.

Edelman, Brooks and Moore have explored the utility of concurrent measurement of nitrogen balance and volume distribution of deuterium oxide to assay change in fat (3). Behnke, Osserman and Welham commented upon the feasibility of checking the validity of such techniques by parallel densitometric analysis (4).

The present study concerns: *1*) evaluation of the use of measurement of nitrogen balance and total body water to estimate change in body fat; and *2*) comparison of the loss or gain in fat so determined with changes obtained by parallel densitometric analysis.

MATERIALS AND METHODS

Subjects of the study consisted of five men and three women. Three were obese, one was normally constituted and the others had several types of metabolic disease (Cushing's syndrome in two, myxedema in one and cirrhosis with malnutrition and ascites in the other). Studies were conducted under conditions of metabolic balance for periods ranging from 20 to 158 days, change in fat (Δ fat) being calculated from the beginning to the termination of each study. Δ Fat was calculated by two independent methods: *1*) Body density (D) and total body water (TBW) were measured, respectively, by underwater weighing and volume distribution of antipyrine or D_2O as described previously (5, 6). Antipyrine was used in Cases 4, 5, 6 and 8 (Table I). Both D_2O and antipyrine were used to measure body water in Cases 1, 2, 3 and 7. The volume distribution of D_2O was in general slightly greater than that of antipyrine. When both were utilized the average of all determinations was used to calculate TBW. These values were substituted into the fat prediction equation (7):

$$\% \text{ fat} = \left(\frac{2.118}{D} - 0.780\ W - 1.354\right) \times 100;$$

where D is body density and W is body water expressed as per cent of body weight. Calculation of total body fat (TBF) by this method at the beginning and end of each study permitted estimation of Δ fat. This method of study will be termed "densitometric."

2) The second method of calculation was that outlined by Edelman, Brooks and Moore (3), *i.e.*, subtraction from change in total body weight (ΔTBwt) of the sum of the changes in the major mobile body constituents, protein and water:

$$\Delta \text{ fat} = \Delta \text{ TBwt} - (\Delta \text{ TBW} + \Delta \text{ protein}).$$

Δ Protein was determined by nitrogen balance by means of the conversion factor of 6.25.[1] The rationale of this

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[1] Although Edelman and his associates (3) express the tissue nitrogen factor as a higher value, 6.25 is used here in accordance with older concepts and because of its use in the equations for fat estimation derived from densitometric and volume distribution techniques. Furthermore, in the type of study reported here loss or gain of labile protein stores would be anticipated; and in such instances the lower nitrogen factor would appear reasonable.

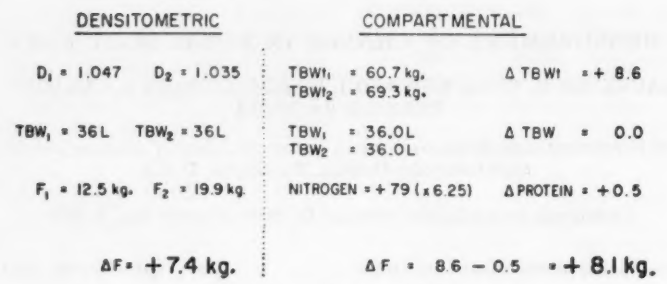
DENSITOMETRIC
COMPARTMENTAL
D_1 = 1.047 D_2 = 1.035
TBW_1 = 36L TBW_2 = 36L
F_1 = 12.5 kg. F_2 = 19.9 kg.
ΔF = +7.4 kg.
$TBWt_1$ = 60.7 kg.
$TBWt_2$ = 69.3 kg.
Δ TBWt = + 8.6
TBW_1 = 36.0L
TBW_2 = 36.0L
Δ TBW = 0.0
NITROGEN = + 79 (x 6.25)
Δ PROTEIN = +0.5
ΔF = 8.6 − 0.5 = + 8.1 kg.

FIG. 1. EXAMPLE OF FAT GAIN

method of measurement rests on the facts that turnover of the mineral phase of the body results in minimal change in weight (from 350 to 500 Gm. with loss or gain of 10 per cent of body stores of ash) and that body carbohydrate is inconsequential in amount and subject to limited variation. Consequently, change in body weight is governed almost entirely by loss or gain of fat, water and protein. This method of measuring Δ fat will be termed "compartmental."

The method of measuring nitrogen balance has been reported from this laboratory (6). Calculations of the assumed error of the methods were made as outlined by Siri (7).

RESULTS

Figure 1 demonstrates the method of calculation and changes in total body fat as determined by densitometric and compartmental techniques in a normally constituted man (Case 1, Table I) in whom weight gain of 8.6 Kg. was encouraged, over a study period of 105 days, by administration of cortisol. Densitometric analysis indicated a gain of 7.4 Kg. and compartmental measurement indicated a gain of 8.1 Kg. of total body fat. Figure 2 depicts similar calculation of Δ fat in an obese man (Case 2, Table I) who lost 27.0 Kg. of weight during 50 days of stringent caloric restriction. Densitometric measurement indicated a loss of 15.3 Kg. of fat, whereas by compartmental analysis the loss of fat was 11.8 Kg. The substantial difference in Δ fat calculated by the two methods was less than the possible error of the methods, 4.2 Kg., necessarily large in this very obese subject.

Table I summarizes the salient data in the eight studies. There were no significant differences in measurements of body fat obtained by the two methods ($p > 0.90$). Comparison of individual studies revealed that differences between results of the two methods were well within the assumed systematic error of fat estimation, with one exception which will be discussed below.

Table I also summarizes the differences between densitometric and compartmental estimation of Δ fat expressed as per cent of mean total body fat and as per cent of mean change in fat. Differences averaged 2.9 per cent of total fat and 10.7 per cent of the mean change in fat.

DISCUSSION

While measurement of body volume and subsequent calculation of body density has permitted crude but useful division of the body into fat-free

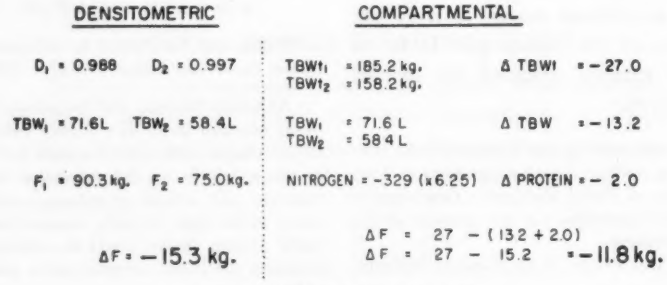
DENSITOMETRIC
COMPARTMENTAL
D_1 = 0.988 D_2 = 0.997
TBW_1 = 71.6L TBW_2 = 58.4L
F_1 = 90.3 kg. F_2 = 75.0 kg.
ΔF = −15.3 kg.
$TBWt_1$ = 185.2 kg.
$TBWt_2$ = 158.2 kg.
Δ TBWt = −27.0
TBW_1 = 71.6 L
TBW_2 = 58.4 L
Δ TBW = −13.2
NITROGEN = −3.29 (x 6.25)
Δ PROTEIN = − 2.0
ΔF = 27 − (13.2 + 2.0)
ΔF = 27 − 15.2 = −11.8 kg.

FIG. 2. EXAMPLE OF FAT LOSS

TABLE I

Comparison of Δ fat as determined by densitometric and compartmental methods

CASE & SEX	CLINICAL PROBLEM	DAYS of STUDY	Δ IN FAT (Kg) I DENSITOMETRIC (D+TBW)	Δ IN FAT (Kg) II COMPARTMENTAL (TBW+ NIT BAL)	DIFFERENCE I-II (Kg)	CALCULATED TOTAL POSSIBLE ERROR (Kg)	DIFFERENCE I − II % OF MEAN TOTAL BODY FAT	DIFFERENCE I − II % OF MEAN CHANGE in FAT
1 ♂	NORMAL	105	+7.4	+8.1	+0.7	(1.7)	4.3	9.0
2 ♂	OBESITY	50	−15.3	−11.8	−3.5	(4.2)	4.2	26.0
3 ♀	OBESITY	28	−3.9	−3.9	0.0	(2.7)	0.0	0.0
4 ♂	OBESITY	20	−1.8	−1.7	−0.1	(4.1)	0.1	5.7
5 ♀	CIRRHOSIS MALNUTRITION	38	−6.8	−7.2	+0.4	(1.7)	3.1	5.7
6 ♂	MYXEDEMA	41	−1.9	−2.2	+0.3	(2.3)	0.8	14.5
7 ♀	CUSHING'S SYNDROME	27	+6.5	+6.8	+0.3	(1.3)	1.7	4.5
8 ♀	CUSHING'S SYNDROME	158	−12.7	−15.6	+2.9	(1.8)	8.9	20.0
					$p > 0.90$		AV.= 2.9%	AV.= 10.7%

and lipid components, this basic technique has numerous disadvantages. Measurement of body volume by underwater weighing is difficult and time consuming in all subjects, impossible in the bedridden patient. Utilization of the helium concentration technique of Siri (8) may solve the problem of conducting densitometric measurement in patients who lack the stamina necessary for underwater weighing, but scarcity of facilities for this type of measurement prohibits general clinical application. While abnormal hydration may be partly circumvented by measurement of total body water, deviation of density of the fat-free body (lean body mass) from the assumed norm constitutes a major impediment to densitometric quantitation of total body fat. Fortunately, in the type of study reported here, abnormality of the fat-free body density introduces minimal error when successive densitometric measurements are conducted in the same person to calculate Δ fat. Despite these multiple limitations, densitometric evaluation of body fat, when conducted under the proper conditions and with suitable correction of abnormal hydration, is the most accurate method for measurement of gross body composition and serves as the best standard of reference for evaluating other techniques of fat estimation. As emphasized by Siri, however, increased accuracy of fat measurement is contingent upon development of methods independent of densitometry (8).

The data reported here indicate that concurrent measurement of nitrogen balance and TBW serves as a fairly simple and, in terms of the densitometric reference, quite accurate technique for evaluation of change in body fat. Use of TBW measurements to correct densitometric analysis and to calculate change in fat by compartmental methods would obviously tend to dampen differences between the two methods; but the complete independence of the techniques is equally apparent. Two of the studies demonstrated considerable differences between the two methods in their measurements of total fat change and these studies deserve further comment.

In Case 2 the patient was an extremely obese man whose body density was 0.988 at the beginning and 0.997 at the end of 50 days of a

restricted caloric intake. Δ Fat calculated densitometrically was −15.3 Kg., whereas by compartmental analysis loss of fat was 11.7 Kg. Considering, however, that adipose tissue contains approximately one-fifth of its volume as water, as well as a small amount of nitrogenous tissue, it would appear that compartmental analysis would, with significant fat loss in extremely obese subjects, indicate a lesser degree of loss than that demonstrated by densitometric techniques. In Case 2 rough calculation indicates a possible overestimate of fat loss by densitometry, approaching 2.0 Kg., with consequently a true difference between the two techniques of fat estimation, approximating 1.5 rather than 3.5 Kg. Calculation of the density of the lost tissue

$$\left(\frac{\Delta \text{ body weight}}{\Delta \text{ body volume}}\right)$$

which was 0.941 in this subject, provides some validation of these assumptions.

In the other instance in which there existed discrepancy between the two methods (Case 8), factors were present that could distort fat estimate obtained by densitometric techniques. This patient had Cushing's syndrome and measurements were made before, and for several months following, subtotal adrenalectomy. Gradual correction of abnormal hydration and change in the density of the fat-free body of this protein and mineral depleted patient would be expected to induce error in the densitometric estimation of Δ fat. Furthermore, the length of this study (158 days) might result in a considerable cumulative error in nitrogen balance.

Although a real need exists for more precise methods of fat measurement than are provided by either densitometric or compartmental analysis, the data presented here indicate the utility of both techniques. Whereas densitometric analysis requires a relatively healthy, intensely cooperative patient and specialized facilities, measurement of fat change by determinations of nitrogen balance and total body water is comparatively simple and applicable in any metabolic unit. This admittedly gross technique provides the opportunity to assess many aspects of fat metabolism that have been poorly explored and permits the accumulation of needed data on the loss or gain of this important body constituent.

SUMMARY

Comparison was made of two independent methods of estimating body fat changes in eight patients studied under conditions of metabolic balance over periods from 20 to 158 days. Serial measurements of body fat were obtained by substituting values for body density (underwater weighing) and total body water into a single fat prediction equation. Change in total body fat, which ranged from a gain of 7.4 Kg. to a loss of 15.3 Kg., as determined densitometrically, was compared with variations in fat as determined by subtracting the sum of measured changes in protein and water from change in body weight. In the eight studies there was no significant difference in calculated Δ fat as determined by the two methods ($p > 0.90$). Comparison of individual studies revealed differences that fell within the potential error of the methods. The close correlation between the two methods strengthens the validity of both and indicates that estimation of change in total body fat can be approached by readily available volume distribution and balance techniques.

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STUDIES ON HEMOGLOBIN METABOLISM. I. THE KINETIC PROPERTIES OF THE PLASMA HEMOGLOBIN POOL IN NORMAL MAN *

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The site of destruction of normal red cells and the subsequent fate of the liberated hemoglobin prior to its degradation is not known. Intravascular red cell rupture or phagocytosis of the red cells by the reticuloendothelial cell system or a combination of these mechanisms are the possibilities usually considered. There is some evidence to show that in some hemolytic disorders at least part of the destruction of the red cells occurs by phagocytosis. This is suggested by histological demonstration of erythrophagocytosis, the specific uptake of red cells in certain organs and the effect of splenectomy in many cases.

There are reasons to believe that the hemoglobin molecules are not reutilized to any great extent (1–4) and, since the days of Aschoff, Whipple and Mann, the reticuloendothelial cells have generally been held responsible for the breakdown of the heme moiety of the hemoglobin molecule. The chief organ site of hemoglobin breakdown is not known, although liver, spleen and bone marrow have been shown to produce bile pigments. The problem has recently been reviewed by With (5).

Hemoglobin is probably normally present in plasma in small amounts, but the turnover of the plasma hemoglobin pool is not known. Since about 1 per cent of the circulating hemoglobin mass is renewed daily, one should expect the turnover of the plasma hemoglobin to be about the same, or 6 to 7 Gm. per day, if the destruction of red cells only takes place intravascularly. The same result should be expected if the red cell breakdown occurred extravascularly and the liberated hemoglobin had to be processed at sites other than those of liberation, although this latter possibility is less likely. On the other hand, if the plasma hemoglobin turnover is found to be negligible compared to 6 to 7 Gm. per day, then the possibility of intravascular breakdown cannot be seriously considered.

The present work is an attempt to define quantitatively the kinetic properties of the plasma hemoglobin pool and to evaluate the importance of this "intermediate" as a pathway in normal hemoglobin metabolism. We shall be concerned with the properties of the pool only when its hemoglobin content is below the haptoglobin binding capacity. Laurell and Nyman (6) and Jandl, Jones and Castle (7) have recently published data on the disappearance of hemoglobin from plasma in man. Their data will be discussed in the light of the present work.

THEORETICAL CONSIDERATIONS

We shall assume that the plasma hemoglobin compartment represents a pool interposed between an inflow of hemoglobin, J_{in}, and an outflow, J_{out}. The concentration of hemoglobin in plasma is c and the volume of the plasma is V. The stationary condition is then given by:

$$\frac{dcV}{dt} = J_{in} - J_{out} = 0. \qquad 1)$$

A stationary state obeying Equation 1 can exist for any number of values of c. There will also exist a number of nonstationary states for which we have:

$$\frac{dcV}{dt} = J_{in} - J_{out} \neq 0. \qquad 2)$$

Our purpose is then to evaluate the outflow for various values of c. We may reasonably suspect that the inflow, J_{in}, is independent of the concentration, c. This will provide a complete phenomenological description of the kinetics of the pool. During the course of the analysis, a specific model will be treated. The observed experimental data are in accordance with this model.

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The simplest case, where we assume that the outflow is directly dependent on the concentration is:

$$\frac{dcV}{dt} = -k \cdot c + J_{in} \qquad 3)$$

which can be ruled out on the basis of the data of Laurell and Nyman, who found an almost constant rate of elimination of injected hemoglobin between concentrations of 100 to 20 mg. per 100 ml. plasma. Jandl, Jones and Castle performed similar studies and followed the plasma concentrations down to lower values. Their data indicate a relatively constant rate of elimination at high levels, but at low levels the transfer rates decreased.

The simplest model consistent with these reported data is one in which it is assumed that the hemoglobin, during the processes responsible for its removal, enters into a series of reactions where the steady state overall reaction rate approaches a limit as c increases. From measurements of the overall reaction rate in the stationary state (J_{out}) and the concentration c, little can be learned about the details of the mechanism of the removal. However, since it will be shown that the data obtained are consistent with one rather reasonable and not too general model, this will be treated below. The hemoglobin molecules are supposed to enter into a reaction with a component or catalyst that is conserved, *i.e.*, regenerated in some later reaction step. This first reaction may be one of physical adsorption or chemical complexing, perhaps on the surface of cells responsible for the removal. After an arbitrary number of consecutive reversible reaction steps, there follows a reaction that is practically irreversible and in which the hemoglobin is disposed of and where the catalyst is regenerated. In such a situation it can be shown (8) that the steady state overall reaction rate, in this case J_{out}, will depend on the concentration, c, in the following simple manner:

$$J_{out} = \frac{k_1 \cdot c}{k_2 + c} \qquad 4)$$

where k_1 and k_2 are constants which in a complicated way (see Reference 8) contain the concentrations of all reactants and products, and the rate constants for their formation and degradation.

Inserting this expression for J_{out} in Equation 2 we obtain:

$$\frac{dcV}{dt} = J_{in} - \frac{k_1 \cdot c}{k_2 + c} \qquad 5)$$

and, with the assumption that J_{in} is independent of c, using Equation 1;

$$\frac{dcV}{dt} = \frac{k_1 \cdot c_n}{k_2 + c_n} - \frac{k_1 \cdot c}{k_2 + c} \qquad 6)$$

where c_n is the normal concentration.

Equation 6 can be integrated to yield measured values of c with respect to time in terms of the constants. If c_n and V are known or measured, the constants can be evaluated by experiments in which hemoglobin is injected and its concentration determined at various time intervals. Although this approach will, in principle, be sufficient for a determination of the constants k_1 and k_2 and thus for a complete kinetic description of the system, it is clear from the integrated form of Equation 6 that the values of the constants will be critically dependent on differences between measured values of c and c_n. These differences become small when the normal concentration is approached and their error can be expected to be large.

Another way of evaluating the constants is by way of a tracer experiment using labeled hemoglobin. In principle, this has the advantage of being more accurate at low concentrations. Assuming that no interfusion occurs, the tracer equation is:

$$-c \cdot \frac{1}{c'} \cdot \frac{dc'V}{dt} = \frac{k_1 \cdot c}{k_2 + c} \qquad 7)$$

where c' is the concentration of the labeled hemoglobin; giving

$$-\frac{d\ln c'}{dt} = \frac{1}{V} \cdot \frac{k_1}{k_2 + c} \quad \text{and} \quad -\frac{\Delta \ln c'}{\Delta t} = \frac{1}{V} \cdot \frac{k_1}{k_2 + c_a} \qquad 8)$$

where c_a is the average concentration during Δt. The latter integration is valid for $\Delta c' \gg \Delta c$. In a true tracer experiment, c is, of course, constant and $d\ln c'/dt = \text{constant}$.

It should be noted that the expression,

$$-V \cdot c_n \cdot \frac{\Delta \ln c'}{\Delta t} \qquad 9)$$

is a measure of the flow J_{out} at the particular value of $c = c_a$ and that this measure is independent of any proposed model for the system.

Although, in general, either the nonstationary approach or the tracer approach should suffice for a complete description, it will be shown that the data are too variable to obtain the information by using one method alone. Using both approaches, however, reasonably accurate estimates of the constants can be obtained.

MATERIALS AND METHODS

Twelve healthy, adult subjects between 20 and 30 years of age, 11 male and one female, were injected intravenously with a known amount of an *in vivo* labeled hemoglobin solution and venous blood samples were subsequently drawn for determination of radioactivity. The experiments were performed in the morning and the subjects were not fasting. They were allowed to move freely about between samplings.

Preparation of in vivo *labeled hemoglobin solution.* A 32 year old white male with polycythemia vera and in good general condition was injected twice intravenously with a ferric citrate solution containing a total amount of about 0.7 mg. of iron and about 1.2 mc. of Fe^{59}. Three to five weeks after the injection venous blood was withdrawn and hemoglobin solutions were prepared by freezing and thawing. The hemolysates were diluted and Seitz-filtered. The final solutions used for injection into the recipients contained 22 to 38 mg. per ml. of hemoglobin and 20,000 to 30,000 cpm per ml. of Fe^{59}. The amount of hemoglobin given was 1.3 mg. per Kg. body

weight of hemoglobin in nine subjects and 3.5 mg. per Kg. body weight in three subjects. Aliquots of the solutions were taken and counted with the samples. The hemoglobin was determined spectrophotometrically as oxyhemoglobin by the Evelyn photoelectric colorimeter.

Estimation of plasma hemoglobin radioactivity. The plasma hemoglobin was isolated by precipitation of the proteins in acid solution, which leaves essentially all of the transferrin iron in the supernatant. To 8 to 10 ml. of plasma, 4 ml. of 2 N HCl and 4 ml. of 20 per cent trichloracetic acid were added and the mixture stirred with a thin glass rod. The precipitate containing hemoglobin and the supernatant containing transferrin iron were separated by centrifugation. Each was wet-ashed in Kjeldahl tubes with sulphuric acid and perchloric acid. After digestion, the samples were electroplated according to the method of Peacock and associates (9). Counting was performed using a helium-filled Geiger-Müller tube. Recovery experiments revealed that about 1 per cent of added hemoglobin activity was lost to the supernatant. About 3 per cent of added radioactive transferrin was lost to the precipitate. Corrections for inefficient precipitation and coprecipitation were performed using these figures.

RESULTS

The results of the measurements of the plasma hemoglobin radioactivity as a function of time are shown in Figure 1. It is evident that the disappearance of the tracer follows relatively closely a first order reaction over the range that was observed. As the labeled hemoglobin was not injected in a true tracer dose, the slope of the disappearance curve should be slightly larger for smaller values of the hemoglobin concentration. A tendency for this can be seen in Figure 1 and is substantiated by the fact that there is a significant negative correlation (coefficient of correlation $= -0.62$) between the individual values of $\Delta \ln c'/\Delta t$ and c_a. The values of c_a were obtained from the number of cpm found in the plasma and the known number of cpm per mg. hemoglobin injected and a value of 0.0042 mg. per ml. added as representative for the normal hemoglobin concentration (see below).

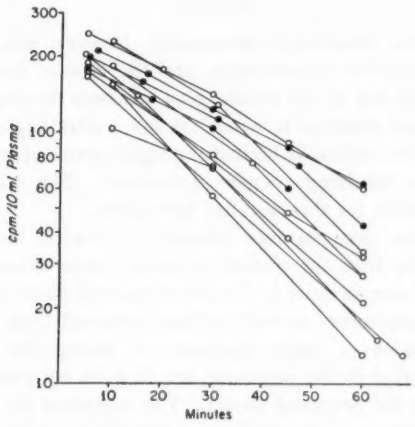

FIG. 1. THE TIME COURSE OF THE RADIOACTIVITY FROM Fe^{59} HEMOGLOBIN INJECTED AT ZERO TIME IS SHOWN

The dose was 1.3 mg. of hemoglobin per Kg. body weight (○) and 3.5 mg. per Kg. body weight (●).

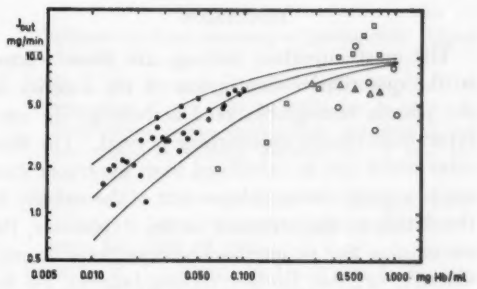

FIG. 2. THE RELATION BETWEEN THE FLOW OF HEMOGLOBIN OUT OF PLASMA, J_{out}, AND THE PLASMA HEMOGLOBIN CONCENTRATION c

○ and △ from data of Laurell and Nyman (6); □ from data of Jandl, Jones and Castle (7); ●, present experiments. Curves represent Equation 4 with values of constants as given in text.

In Figure 2, the flow J_{out} is plotted against the concentration c. The tracer data were obtained from Expression 9 with a plasma volume of 3,000 ml. The data of Laurell and Nyman (6) and Jandl, Jones and Castle (7) were used to give flows for average concentrations by calculating the quotient:

$$\frac{c_{t_1} - c_{t_2}}{t_2 - t_1} \text{ for } J_{out} \text{ and the expression } \frac{c_{t_1} - c_{t_2}}{2} \text{ for } c.$$

It is evident from the figure that the relation between the outflow and the concentration is similar to one predicted by the theory. The curves are drawn by using the following values of the constants in Equation 4: upper curve $-k_1 = 10$, $k_2 = 0.04$; middle curve $-k_1 = 10$, $k_2 = 0.07$; lower curve $-k_1 = 10$, $k_2 = 0.14$.

DISCUSSION

The most important findings are those related to the quantitative description of the kinetics of the plasma hemoglobin pool at hemoglobin concentrations closely approaching normal. The flow rates which can be calculated from the tracer data are to a great extent independent of the validity of the details of the proposed model. However, the assumption that no interfusion occurs must be considered; *i.e.*, that isotopic mixing between the intravascular and extravascular hemoglobin is negligible compared to the net movement of the hemoglobin. This phenomenon can be evaluated by a comparison of the disappearance kinetics of hemoglobin and albumin. Studies on the latter compound have revealed (10) that less than about 5 per cent will have left the plasma one hour after the injection. This figure is a maximum estimate for interfusion disappearance of albumin; since the haptoglobin-hemoglobin complex is considerably larger than the albumin molecule, the interfusion disappearance of the former must be even smaller. Thus, interfusion does not play a significant role in the disappearance of labeled hemoglobin from plasma and it is reasonable to assume that specific cellular mechanisms exist for the transfer.

The present data, covering plasma concentration values down to the normal range, are of particular interest in connection with the problem of the normal turnover of plasma hemoglobin. Hanks and Chaplin (11), using a modified benzidine method, have recently found the normal value of the plasma hemoglobin concentration to be 0.42 mg. per 100 ml. (range 0.25 to 0.58 mg. per 100 ml.), substantially lower than earlier estimates. If this value is accepted, the present data (Figure 2 and Equation 4 with its constants) indicate that normally only about 0.8 Gm. of hemoglobin flows in and out of the plasma compartment each day. This value corresponds to only slightly more than 10 per cent of the overall red cell hemoglobin breakdown and indicates that only a small part of the normal red cell destruction occurs intravascularly.

In a variety of hemolytic disorders Crosby and Dameshek (12) found values considerably above the normal value as accepted at that time. Provided that the capacity to remove hemoglobin from plasma is essentially normal in these cases, quite a considerable intravascular hemolysis must have occurred. Thus, in a patient with acquired hemolytic anemia, where the destruction is about six to eight times normal and the plasma hemoglobin level about 15 mg. per 100 ml., approximately twice the normal overall destruction would occur intravascularly and about three to four times the normal overall destruction would occur extravascularly. It is of considerable interest to note that the recent investigations concerning the behavior of the haptoglobin (6, 13, 14) in various conditions are probably best interpreted in favor of increased intravascular hemolysis in hemolytic disorders.

The data presented here suggest that an adsorption process, whether the binding energy is small as in "physical" adsorption or large as in "chemical" adsorption, is intimately linked with the removal process. It should, however, be pointed out that the fact that the data are in accordance with the proposed simple model does not imply that this model necessarily exists. Several different, but physically more complicated, models might fit the data as well or better. It is, however, felt that as long as the simplest model fits the data within their accuracy, this model should be retained as a working hypothesis.

SUMMARY

The theoretical relationship between plasma hemoglobin concentration and the flow of hemoglobin out of the plasma pool has been developed for the situation in which the rate controlling step for the outflow is limited through a series of reactions involving complex formation. The tracer equation for this case has been given.

The disappearance kinetics of labeled hemoglobin from the plasma in nearly tracer amounts has been measured. The data obtained from these measurements as well as those obtained from the behavior of larger amounts of hemoglobin, as reported in the literature, are in good accordance with the proposed model. The constants for the prediction of the outflow of hemoglobin from the plasma for various concentrations of plasma hemoglobin are given.

The data indicate that the plasma hemoglobin is a relatively unimportant pathway in normal

hemoglobin metabolism and that a few red cells die intravascularly.

ACKNOWLEDGMENTS

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STUDIES ON HEMOGLOBIN METABOLISM. II. PATHWAYS OF HEMOGLOBIN IRON METABOLISM IN NORMAL MAN *

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It has been suggested by earlier work (1, 2) and especially by a recent paper by Finch (3), that when isotopically labeled iron is administered to the body the time required for complete isotopic equilibrium to be reached is very long, a matter of years. It is well established that labeled iron bound to transferrin is found almost entirely in the red cells two to three weeks after injection. These findings could well be explained on the basis of poor exchangeability of the storage iron, since lattice-bound atoms of such a semicrystalline structure (4, 5) are in general very firmly bound. An alternative explanation is that storage iron is largely bypassed in the normal endogenous cycling of iron. First, it is quite possible that at least part of the products of red cell degradation are specifically taken up by cells of the bone marrow such as reticuloendothelial cells and red cell precursors (6), and in this manner exclude major participation by storage iron in heme formation. Second, the red cell iron compartment has the properties of a large nonexchangeable pool interposed between two one-way flows with a relatively fixed time (about 120 days) spent by the isotope in the pool. For this reason alone isotopic equilibrium within the body will take a very long time.

The present investigation was undertaken to define more exactly the pathways of iron following the degradation of hemoglobin when this was administered intravenously in amounts comparable to physiological loads. In normal man, the total hemoglobin turnover is about 6 to 7 Gm. per day or about 270 mg. per hour. The present data were obtained after injection of 100 to 250 mg. of hemoglobin. The reappearance of the labeled iron in plasma, presumably as transferrin iron, and the subsequent incorporation of this iron into circulating red cells was studied.

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MATERIALS AND METHODS

In a previous paper (7) the preparation of an Fe^{59} tagged hemoglobin solution and its intravenous injection in 12 healthy, adult, volunteer subjects was described. The present data are based upon further observations on these subjects and one additional subject. The time course of the radioactivity of the nonhemoglobin, easily hydrolyzable iron in the plasma was followed. The time course of the radioactivity of the red cells was also followed. The wet-ashing and counting procedures were identical to those described in a previous paper (7). Hematocrits were determined according to Wintrobe (8). Estimations of the red cell volume and the plasma volume were made using a value of 29 ml. per Kg. body weight for red cell volume and a body hematocrit of 0.92 times the venous hematocrit. The systematic error introduced by such a calculation is unlikely to exceed 10 per cent for the red cell volume and 20 per cent for the plasma volume, and probably does not exceed 5 and 10 per cent, respectively (9).

RESULTS

The time course of the radioactivity in the nonhemoglobin, easily hydrolyzable iron in the plasma after injection of labeled hemoglobin is shown in Figure 1. The hemoglobin injection was made at zero time and the amount of hemoglobin injected was 1.3 mg. per Kg. body weight in 10 subjects and 3.5 mg. per Kg. body weight in three subjects [compare with previous paper (7)]. No significant difference was found in the curves of the different loads. The interrupted line represents the fraction of the injected dose which was split off from hemoglobin and transported into the plasma. This curve was obtained from the continuous line by adding graphically the cumulative amounts which left the plasma each

time. The procedure utilized the fact that the disappearance of about 90 per cent of labeled transferrin iron from the plasma can be described adequately by a single exponential with the coefficient of transfer of 0.45 per hour (10, 11) and a standard error of 0.04 per hour.

The time course of the radioactivity from Fe^{59} in circulating red cells is shown in Figure 2. Not included in this figure are 10 additional values obtained between 54 and 69 days after the injection, from which a mean value of 107 per cent (S.E. = 7 per cent) was calculated.

DISCUSSION

The time course of the appearance of the labeled iron in plasma after injection of hemoglobin showed that about 30 per cent of the injected activity was released and passed into the plasma after about two hours; another 10 per cent was passed during the next six hours. It is thus evident that plasma hemoglobin rapidly enters the catabolic cycle. This type of appearance curve suggested that considerably more iron was released from heme than passed through the plasma. Two possibilities are suggested concerning the fate of the iron which did not pass into the plasma during the time of investigation. The first possibility is that part of the iron was incorporated into slowly turned-over storage compounds in reticuloendothelial cells not located in the bone marrow. The second possibility is that part of the hemoglobin catabolism took place in the bone marrow and that subsequent liberation of iron to the red cell precursors bypassed the plasma. The present data offer no choice between the two possibilities. It is, however, of interest that Mann, Sheard, Bollman and Baldes (12, 13) were able to show higher concentrations of bilirubin in venous blood from the marrow than in arterial blood and that they concluded the bone marrow was an important site of hemoglobin breakdown.

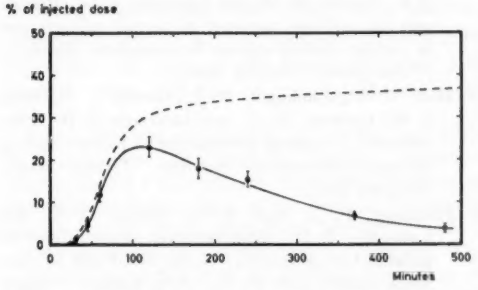

FIG. 1. THE TIME COURSE OF THE RADIOACTIVITY (Fe^{59}) IN THE NONHEMOGLOBIN IRON IN THE PLASMA AFTER INJECTION OF LABELED HEMOGLOBIN AT ZERO TIME

Mean values ± S.E. in 13 experiments.

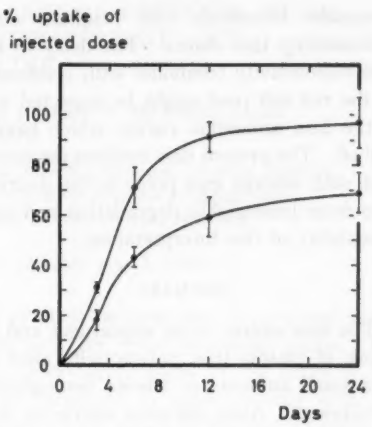

FIG. 2. THE TIME COURSE OF THE RADIOACTIVITY (Fe^{59}) IN CIRCULATING RED CELLS AFTER INJECTION OF LABELED HEMOGLOBIN AT ZERO TIME

Mean values ± S.E. in 13 experiments. The upper curve represents values (mean ± 2 S.E.) from injection of labeled transferrin (3).

The time course of the uptake of labeled iron into circulating red cells showed a smaller proportion of the injected activity in the red cells at any time than after injection of transferrin-bound iron. This is in accordance with the idea that at least part of the iron from hemoglobin is incorporated into relatively inaccessible iron compounds. The turnover of this pool, however, was sufficiently rapid to allow most of the injected hemoglobin iron to be reutilized within two months after injection.

It has been shown that in various types of hemolytic disorders, the rate of uptake of radioiron in the circulating red cells after intravenous injection of Fe^{59} bound to plasma transferrin and the subsequently reached plateau is lower than in normals (10, 14–16). This may be interpreted as the result of the very early destruction of young red cells and the degradation of their hemoglobin in the reticuloendothelial system and/or increased

intravascular hemolysis with resultant shunting of radioactivity into stores. The delay in release of this radioactivity combined with continual loss from the red cell pool might be expected to produce the iron utilization curves which have been described. The present data confirm the participation of such storage iron pools in the distribution of iron from hemoglobin degradation and support the feasibility of this interpretation.

SUMMARY

1. The time course of the appearance and disappearance of plasma iron radioactivity after injection of small amounts of labeled hemoglobin has been followed. Also, the time course of the appearance of the labeled iron in red cells was investigated.

2. The data show that hemoglobin, injected in amounts of 100 to 250 mg., rapidly enters its catabolic cycle and that at least 40 per cent of the iron is split off within the first few hours after injection. A considerable part of the iron is retained extravascularly for many hours. The subsequent incorporation of the labeled iron into circulating red cells is slower than after administration of transferrin-bound radioiron.

3. The data substantiate the idea that the decreased rate of appearance of radioiron in circulating red cells in certain types of hemolytic anemia may be due to destruction of young cells or increased intravascular hemolysis and subsequent shunting of radioiron into slowly turned-over iron stores.

ACKNOWLEDGMENTS

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PERSISTENCE OF ANTIBIOTICS IN BLOOD OF PATIENTS WITH ACUTE RENAL FAILURE. I. TETRACYCLINE AND CHLORTETRACYCLINE * †

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The management of patients with severe renal disease is frequently complicated by infections; these commonly involve the urinary tract, but infections elsewhere are not unusual. Overwhelming infection, particularly with gram-negative bacteria, is often associated with shock and anuria (2) and antibiotic therapy is an integral part of the management in such cases. The persistence of a drug in the blood, once it is absorbed or after it is given systemically, depends on many factors which include: removal by the kidneys, binding to plasma proteins, sequestration in various organs and body compartments, excretion into the bile and feces, detoxification and degradation mechanisms and the inherent stability of the substance; most important for nearly all antibiotics, however, is their removal by the kidneys. Knowledge concerning the fate of antibiotics in patients with impaired renal function is, therefore, necessary for planning therapy so that it will insure adequate blood and tissue levels without undue retention that may be harmful.

A series of studies was, therefore, undertaken in patients with anuria or impaired renal function from various causes, who either were receiving antibiotics as part of their therapy, or were given test doses of some of the commonly used antibiotics. The half-life of the antibiotics in the blood of such patients was determined and correlated with the renal functional status. In addition, since hemodialysis by the artificial kidney may be employed in some patients with anuria who retain toxic drugs or excretory products, the effectiveness of this procedure in extracting antibiotics from the blood of suitable patients was included as part of these studies. Observations on chloramphenicol (3) and certain other antibiotics (4) (penicillin, streptomycin, erythromycin and kanamycin) are reported elsewhere.

The present paper deals with tetracycline and chlortetracycline. Antibiotics of the tetracycline group have shown very little direct toxicity when given in the usual therapeutic doses. However, under certain circumstances they may produce a negative nitrogen balance and increased riboflavin excretion in the urine (5–8) and prolonged therapy with large doses, particularly if administered intravenously, has given rise to changes in morphology and function of the liver (6–11). Impairment of renal function during prolonged, high-dosage therapy with oxytetracycline has also been reported (12). The results to be presented here should prove useful as a basis for selecting a dosage schedule of these antibiotics which would achieve adequate levels of the active drugs in uremic patients similar to those attained with the usual therapeutic doses in patients with normal renal excretory function and thus reduce the hazard of additional toxic effects from these drugs.

MATERIAL AND METHODS

Patients. Thirty-five patients whose renal functional status ranged from normal to protracted anuria due to various causes were studied on the wards of the Boston City and Peter Bent Brigham Hospitals. Anuria is arbitrarily defined, in this study, as the condition in which the patient excreted less than 400 ml. of urine per day

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† An abstract of part of this study has appeared (1).

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TABLE I

Half-life of tetracycline in serum of five patients with acute renal failure

Patient	Age	Sex	Precipitating factor	Urine output	Tetracycline received*		Period of study†	Rate of decay of tetracycline in serum	Half-life of tetracycline in serum
	yrs.			*ml./day*	*Gm.*	*route*	*days*	%/hr.‡	
H	54	M	Hypotension following resection of aortic aneurysm	200–300	4.3	IV	3(*a*)		>3 da.
							5(*b*)	0.64 ± 0.25	108 hrs.
C	49	F	Right nephrectomy, pyelonephritis	< 100	1.2	IM	2½		>2½ da.
S	43	F	Mismatched transfusion	100–300	2.0	IV	5	1.22 ± 0.29	57 hrs.
I	58	M	Postoperative hypotension	100–200	1.0	IV	5	0.69 ± 0.15	101 hrs.
A	72	M	Hepatic abscess and shock	< 100	2.0	IV	3	0.64 ± 0.06	108 hrs.

* Total amount given to patient before start of observations; IV = intravenous; IM = intramuscular.
† Two periods in patient H; (a) before dialysis; (b) after dialysis.
‡ Mean ± 1 standard error.

for three or more days; the eight anuric patients in this study excreted considerably less than this amount. The nonanuric patients had chronic glomerulonephritis, pyelonephritis or arteriolar nephrosclerosis. Two of the patients were twins, one of whom developed uremia from chronic glomerulonephritis, and was the recipient of a healthy kidney from the other, the donor, who was studied following removal of one of his kidneys. Among the control patients were a few otherwise normal individuals who had diminished creatinine clearance of undetermined etiology; some of them were elderly patients.

The severity of renal impairment in each subject was determined by the 24 hour endogenous creatinine clearance test. A modification of the method of Bonsnes and Taussky (13) was used for determining creatinine in blood and urine. The anuric patients were arbitrarily assigned a creatinine clearance value of less than 1 ml. per minute.

Hemodialysis. The blood of six subjects was subjected to dialysis through the artificial kidney during the course of this study; a rotating drum apparatus (Kolff-Brigham type (14) was used in two patients and a Kolff-coil type (15) was used in the others. Determinations of tetracycline, chlortetracycline and creatinine were carried out on arterial and venous samples entering and leaving the artificial kidney, respectively. Levels of tetracycline in the venous blood of the patients prior to and on completion of dialysis were also determined. The extraction ratio (ER) of the substances was obtained by the formula:

$$ER = \frac{A - V}{A} \times 100,$$

where A and V represent the concentrations of the substance entering and leaving the device, respectively.

Serum levels and half-life of tetracyclines. A dose of 500 mg. of tetracycline hydrochloride [1] or chlortetracycline hydrochloride [1] in 150 ml. of 5 per cent dextrose in water was administered intravenously over a period of 15 to 30 minutes to most of the subjects. Five subjects received a similar dose orally and are considered separately since continued absorption could not be excluded. A few of the patients who were anuric or severely uremic were given tetracycline in larger doses by the intravenous route for therapeutic purposes. Blood was obtained two hours after the intravenous dose or six hours after the oral dose and then at intervals of two to 24 hours depending on the severity of renal impairment; a minimum of four specimens was obtained in each subject, and six to eight specimens were procured in most of them. The sera were separated as soon as possible and stored at − 20° C. Concentrations of tetracycline and chlortetracycline were determined by the serial twofold dilution method in brain heart infusion broth (Difco pH 7.4 per cent) using *B. cereus* No. 5 as the test organism, 0.5 ml. of a 10^4 dilution of the fully grown culture being added to an equal volume of the serum dilutions. In a few instances, the cup-plate assay method was also employed; [2] all serum levels in the hemodialysis experiments were determined by this method.

The method of least squares was used to calculate the slope of the tetracycline decay curve from which the half-life was determined (16). The standard error of each slope was also calculated. Care was taken, when feasible, to obtain sera at intervals greater than the estimated half-life of the antibiotic in order to minimize the error of the assay method. In a previous study (16) it was shown that although the results of the twofold dilution method had a greater standard deviation and were generally lower than those of the cup-plate method, the serum half-lives determined by either method were in close agreement.

RESULTS

Tetracycline

The half-life of tetracycline in four individuals with normal creatinine clearances given 0.5 Gm. intravenously ranged five to seven and six-tenths and averaged five and eight-tenths hours. The

[1] Supplied as Achromycin® and Aureomycin® by the Lederle Laboratories.

[2] We are indebted to A. C. Dornbush of Lederle Laboratories for the cup-plate assays of the tetracycline antibiotics.

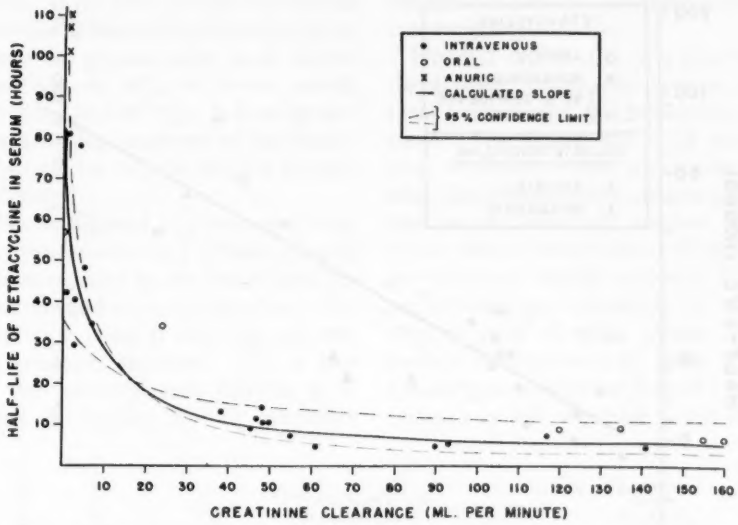

FIG. 1. RELATION OF HALF-LIFE OF TETRACYCLINE IN SERUM FOLLOWING AN INTRAVENOUS DOSE TO THE RENAL FUNCTION AS REFLECTED IN THE CREATININE CLEARANCE

mean half-life of tetracycline in four normal subjects given the same dose orally was seven and two-tenths hours. Table I presents pertinent data in five anuric patients, three of whom subsequently died of their underlying illness. The serum levels of tetracycline in these patients declined slowly. In Patients H and C no decline was detectable during two and one-half and three days of observation, respectively. During longer observation periods in Patient H and also in Patients I

TABLE II

Half-life of tetracycline in serum after an intravenous dose of 500 mg. in nonanuric patients with different clearances of endogenous creatinine

Patient	Age	Sex	Etiology of renal disease	Creatinine clearance	Rate of decay of tetracycline in serum	Half-life of tetracycline in serum
	yrs.			*ml./min.*	*%/hr.**	*hrs.*
Mi	36	M	None	140.8	14.5 ± 1.7	4.8
Kn	39	M	None	117.4	9.1 ± 1.7	7.6
Gl	47	M	None	92.5	10.8 ± 2.0	6.4
Jo	34	M	None	89.8	13.9 ± 0.6	5.0
Tu	16	F	?	60.7	12.2 ± 0.7	5.7
McD	26	M	Unilateral nephrectomy	55.0	8.9 ± 0.4	7.8
Pe	66	M	? Arteriolar nephrosclerosis	49.6	6.5 ± 1.6	10.7
Yo	58	M	?	48.0	6.5 ± 0.8	10.7
Ka	75	M	? Arteriolar nephrosclerosis	47.8	4.8 ± 0.3	14.6
McD	26	M	Recipient kidney transplant	44.8	7.2 ± 0.4	9.6
Do	79	M	?	46.6	5.9 ± 0.7	11.8
Yu	67	M	?	37.8	5.1 ± 0.8	13.6
Li	56	F	?	7.0	2.1 ± 0.5	32.6
Wa	50	M	Polycystic kidneys	5.0	1.5 ± 1.3	47.9
Bu	50	F	Recovery phase, acute renal failure	4.0	0.9 ± 0.5	78.7
Mi	38	M	Polycystic kidneys	3.1	1.7 ± 0.4	41.2
Ne	50	M	Chronic pyelonephritis	2.3	2.4 ± 0.3	29.5
Me	44	F	Chronic pyelo- or glomerulonephritis	1.0	1.7 ± 0.3	42.4

* Mean ± 1 standard error.

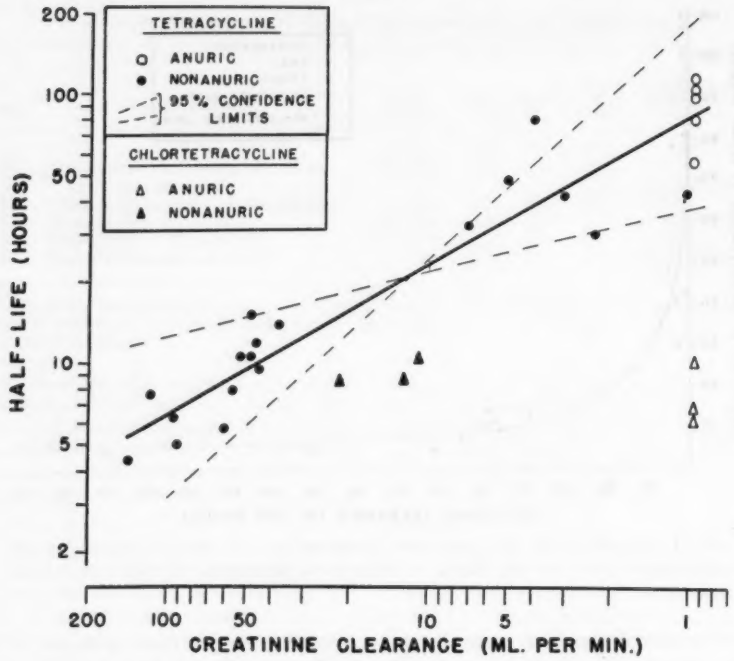

FIG. 2. DATA IN FIGURE 1 PRESENTED ON LOGARITHMIC SCALE
Some observations on half-life of chlortetracycline in serum of three anuric patients and three patients with normal renal function are also included.

and A, the mean half-life was found to be about four to five days. The relatively low value of 57 hours in Subject S will be discussed below.

Figure 1 presents the data for 28 patients in whom simultaneous measurement was made of creatinine clearance and tetracycline half-life. The five anuric patients were arbitrarily assigned a creatinine clearance value of 1 ml. per minute. The serum half-life was similar in all subjects whose creatinine clearance exceeded 60 ml. per minute. As the creatinine clearance fell below this value, the serum half-life of tetracycline rose steeply. The maximum value observed in an anuric patient was 110 hours.

Table II presents data in 17 of the patients depicted in Figure 1. All of them received tetracycline intravenosuly and the half-life of the antibiotic in the serum is shown together with the decay rate of the drug in the serum expressed as per cent fall per hour.

The data in Figure 1 and in Tables I and II may be used to formulate an equation which best expresses the relationship between the two variables, tetracycline half-life and creatinine clearance. The serum half-life may be considered as a geometric function and creatinine clearance as a linear function. A straight line relationship will then be expressed thus: $\log y = mx + b$, where y = tetracycline serum half-life, m = the slope, x = the creatinine clearance and b = the y intercept. When the slope of this line is calculated by the method of least squares, and a correlation coefficient between the two variables is determined, the latter is found to be 0.86. A better fit to the experimental data, however, is provided by the same formula with the creatinine clearance (x) also treated logarithmically; the correlation coefficient then becomes 0.95 and the relationship becomes more linear as shown in Figure 2. The slope of this relationship is 0.55 ± 0.16 and the y intercept is 82 hours. Athough creatinine clearance cannot be considered to be a logarithmic function, neither can it be considered purely linear when used to ascertain glomerular filtration in

uremic subjects. It has been pointed out that the creatinine clearance is less well correlated with the inulin clearance in patients with renal disease than in normal subjects (17), the former usually being somewhat higher (18, 19). It is recognized therefore that a log-log treatment of the data is somewhat arbitrary, but may be justified for purposes of prediction.

The solid line in Figure 1 is a theoretical slope determined by the formula $\log y = \log x + \log 82$ hours. The area enclosed by the broken lines encompasses two standard errors of this slope. The greatest variability is noted at very high and very low levels of creatinine clearance. This in part may explain the relatively short half-life of 57 hours obtained in Subject S. In calculating maintenance therapy, allowance must be made for this variability.

Figure 3 depicts data in a case that illustrates the dynamic relationship between the status of renal function and the tetracycline serum decay curve. The patient was a 23 year old woman who developed severe hypotension following a brisk hemorrhage from a placenta previa. She had been anuric before she received her first intravenous dose of tetracycline. At this time, she began to excrete slightly more than 500 ml. of urine per day and was considered to be in the early diuretic phase of acute tubular necrosis. The levels of tetracycline in the serum fell quite slowly following an intravenous dose of 1 Gm. and tetracycline was still detectable in her serum 10 days

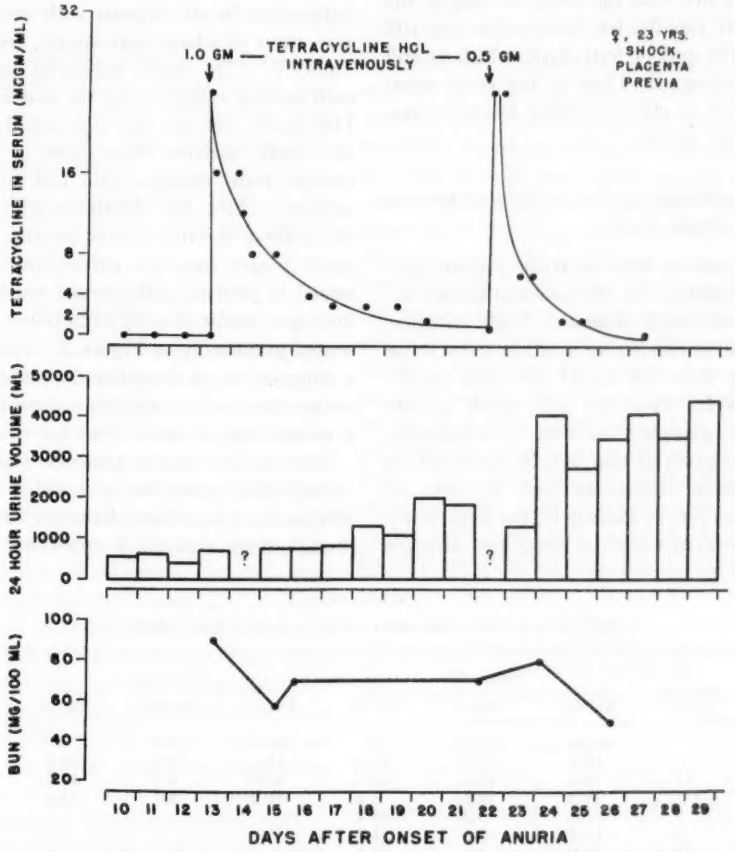

FIG. 3. LEVELS OF TETRACYCLINE IN SERUM FOLLOWING INTRAVENOUS DOSES GIVEN TO A PATIENT DURING THE EARLY DIURETIC AND THE DIURETIC PHASES OF RECOVERY FROM ACUTE RENAL INSUFFICIENCY

TABLE III

Reduction in concentration of tetracycline in blood following dialysis in the artificial kidney

Patient	Type of artificial kidney	Duration of dialysis	Tetracycline in serum		Tetracycline extracted*
			Before dialysis	After dialysis	
		hrs.	*μg./ml.*	*μg./ml.*	*%*
H†	Drum	4	31.0	22.5	27
C	Coil	3½	6.3	5.4	14
S	Coil	4	14.5	12.0	17

* Expressed as per cent of predialysis level.
† In this patient the blood urea nitrogen concentration dropped from 271 to 189 mg. per 100 ml. (30 per cent) during the same period.

later. During the full diuretic phase of her disease, she was given 0.5 Gm. of tetracycline intravenously; after this dose the levels of drug in the serum fell more rapidly, but tetracycline was still detectable at the end of four days, which is still abnormally prolonged. Thus, as her renal status slowly improved, so did her ability to clear tetracycline from her serum.

Effect of hemodialysis by the artificial kidney on tetracycline serum levels

Table III presents data on three patients subjected to hemodialysis by the artificial kidney because of severe renal disease. Each of these dialyses was accompanied by a fall in tetracycline levels ranging from 14 to 27 per cent of the predialysis level. This fall was much greater than would be expected to occur spontaneously, but was not as great as that usually observed for readily dialyzable substances such as urea or creatinine; however, in Patient H, the tetracycline level fell 27 per cent while the blood urea nitrogen fell 30 per cent. Creatinine and blood urea nitrogen values were not obtained immediately prior to and on completion of dialysis on the other two subjects.

Table IV presents data on the extraction ratios observed during six runs performed on three subjects. The extraction ratio of the antibiotic was quite variable, ranging from 5.3 to 24.5 per cent with a mean value of 14.3 per cent; it also varied during different runs in the same patient. The extraction ratio of tetracycline in Patient A also varied with each run and was 21 to 38 per cent of that of creatinine.

Chlortetracycline

Observations on the serum half-life of chlortetracycline in six patients with severe renal disease, three of whom were anuric, are presented in Table V. The mean values of the half-life in each patient ranged from six and eight-tenths to 11.0 hours, but are not significantly different in the anuric patients from those in patients with chronic renal diseases who had better filtration capacity. Also, the chlortetracycline serum half-life in these severely uremic patients was not very much longer than the corresponding values obtained in patients with normal renal function following a similar dose of tetracycline. This is presented graphically in Figure 2. Whereas there is a progressive prolongation in serum half-life of tetracycline as the creatinine clearance falls, such a relationship is not evident for chlortetracycline.

One of the anuric patients (Ken) who received chlortetracycline was subjected to hemodialysis by the artificial kidney (Table VI). Although there was good extraction of creatinine

TABLE IV

Extraction of tetracycline and creatinine by the artificial kidney

Patient*	Sampling period	Tetracycline			Creatinine			$\frac{A}{B}$
		Arterial†	Venous†	ER (A)	Arterial†	Venous†	ER (B)	
		μg./ml.	*μg./ml.*	*%*	*mg. %*	*mg. %*	*%*	*%*
A	I	13.4	12.0	10.4	18.3	9.8	46.5	22.3
	II	12.7	10.7	15.7	13.9	8.2	41.1	38.1
	III	11.2	10.6	5.3	10.9	8.1	25.1	21.1
W	I	6.0	5.2	13.4				
C	I	11.2	8.5	24.5				
	II	10.0	8.4	16.5				

* Subjects A and W were dialyzed on a coil type kidney; Subject C was dialyzed on a rotating drum type apparatus.
† "Arterial" blood represents blood entering the artificial kidney and "venous" blood represents the effluent return.

TABLE V

Half-life of chlortetracycline (CTC) in serum of patients with severe renal disease after an intravenous dose of 500 mg.

Patient	Age	Sex	Type of renal disease*	Precipitating factor for anuria	Urine output	Creatinine clearance	BUN	Decay of CTC in serum	Half-life of CTC in serum
					ml./da.	*ml./min.*	*mg. %*	*%/hr.†*	*hrs.*
Kel	17	M	Anuria	Ethylene glycol intoxication	50–100		67	10.0 ± 0.8	6.9
Ken	35	F	Anuria	Posteclampsia	100–200		117	6.3 ± 0.4	11.0
Wr	21	M	Anuria	Chronic glomerulonephritis	50–100		NPN‡ 146	10.2 ± 0.5	6.8
Ta	70	M	Chronic			22.7	NPN 166	8.3 ± 0.5	8.4
Hu	55	F	Chronic			11.6	NPN 136	6.9 ± 0.3	10.1
Mi	64	M	Chronic			18.2	NPN 70	7.8 ± 0.6	8.9

* The etiology of the renal disease in the nonanuric patients was not known.
† Mean = 1 standard error.
‡ Nonprotein nitrogen.

during the two sampling periods, no detectable decrease in chlortetracycline serum levels was noted. This is in contrast to the findings with tetracycline and suggests that chlortetracycline may be more tightly bound to serum proteins; however, the levels of chlortetracycline in the serum were also much lower when the dialysis was begun in this patient.

DISCUSSION

Although tetracycline and chlortetracycline differ in their structure by only a single chlorine atom and they have very similar therapeutic activity, the data presented in the present report indicates that there is a marked difference in the persistence of these drugs in the serum of patients with renal failure. On the one hand, the serum half-life of active chlortetracycline is very little affected by the presence of uremia, whereas that of tetracycline may increase from six to eight hours, as observed in patients without renal disease, to up to 110 hours in anuric patients. This is not entirely surprising in view of the instability of chlortetracycline in serum or in alkaline aqueous solutions which interfere with certain biologic assays (20, 21). However, when the broth dilution method is employed, as in the present study, and the specimens are assayed together with a suitable standard preparation, the serum decay curve is probably valid.

There is some evidence that chloretetracycline may be more stable *in vivo* than *in vitro*. The studies of Womack and co-workers (22, 23) demonstrated that a fraction of chicken egg yolk exerted a protective effect on chlortetracycline *in ovo* and *in vitro* and studies on the urinary excretion of the drug in man indicated that small amounts of the drug could be recovered in the urine as late as 48 to 72 hours after administration (24–26). The data presented in the present report suggest that there is no great protective effect on chlortetracycline in the uremic patients *in vivo*. The delayed excretion of the drug, noted in other reports, may be explained on the basis of the slow renal clearance and its sequestration and release from certain organs, primarily the liver. This sequestration by the liver has been recently found with oxytetracycline by Leevy, Zinke and Chey (27) and has been observed by Böttiger (28) in mice given chlortetracycline.

TABLE VI

Extraction of chlortetracycline and creatinine by the artificial kidney in patient Ken*

Sampling period	Chlortetracycline			Creatinine		
	Arterial†	Venous†	ER	Arterial	Venous	ER
	μg./ml.	*μg./ml.*	*%*	*mg. %*	*mg. %*	*%*
I	3.6	3.6	0	20.8	10.8	48.1
II	2.4	2.4	0	13.7	9.4	31.4

* A coil type apparatus was used for this dialysis.
† "Arterial" and "venous" indicate blood entering and leaving the device, respectively.

Sirota and Saltzman (29) using the fluorometric assay method, observed that intravenously injected chlortetracycline was cleared by the kidney at about 37 per cent the rate of a simultaneously performed creatinine clearance. These authors concluded from this and from studies of the binding of chlortetracycline by human albumin that the renal clearance of the antibiotic could be explained on the basis of glomerular filtration alone. In a study on the total body and renal clearance of four tetracycline analogues in normal subjects using the cup-plate microbiological assay method, Kunin, Dornbush and Finland (26) found that chlortetracycline was cleared by the kidneys at a rate equivalent to about 30 per cent of a simultaneous creatinine clearance, whereas tetracycline was cleared twice as rapidly, at 62 per cent of the creatinine clearance. The somewhat slower clearance of chlortetracycline noted in the latter study may have been, in part, due to the degradation of the drug during the collection and preservation of the urine (30).

Thus, chlortetracycline appears to be cleared by the kidneys more slowly than tetracycline. That this was not due to retention of active drug in the body is evidenced from the fact that recovery of the former drug in the urine over a period of four days was found to be only about 25 per cent of the amount injected, whereas about 70 per cent of the tetracycline could be recovered in the urine during a similar period (26); much of the difference is probably ascribable to deterioration of the drug *in vivo* (30).

It has been reported that chlortetracycline given by the intravenous route may be recovered from various parts of the intestinal tract(31) and may be found in the bile at levels eight to 16 times that of the serum concentration (32, 33). This, however, will not adequately explain the discrepancy between the half-lives of the two tetracycline analogues in the uremic patients since tetracycline may also be recovered from the bile in appreciable quantities (34, 35). It can therefore be concluded that chlortetracycline is inactivated much more rapidly than tetracycline *in vivo* as well as *in vitro*.

The data presented in the current study were obtained by the twofold dilution method and were thus hampered by the greater inherent error of this method when compared with that of the cup-plate method (16). Thus, the normal values of six to seven hours for the half-life of tetracycline in serum as reported here is somewhat less than the mean of eight and one-half hours observed in another study in which the cup-plate method was used (26). In the latter, the serum half-life of intravenously administered chlortetracycline was five and six-tenths hours although its renal clearance was slower than that of tetracycline. This further supports the inference that chlortetracycline is fairly rapidly inactivated *in vivo*.

The extraction ratios of the two antibiotics across the artificial kidney indicate that both diffuse much more slowly than creatinine, presumably due to their binding to plasma protein. Chlortetracycline, which was not perceptibly altered in the serum by hemodialysis in the one patient studied, presumably is more tightly bound than tetracycline. This was also found in *in vitro* dialysis experiments (26). Thus, the use of hemodialysis in attempts to lower the serum levels of either drug, but particularly chlortetracycline, in patients who are presumed to have inordinately high and possibly toxic levels could not be expected to prove very effective.

On the basis of the observations presented here, the following empirical approach to dosage of tetracycline would appear to be reasonable. Following the administration of a loading dose of 0.5 to 1.0 Gm. orally or intravenously, 0.5 Gm. should be given every six or eight hours for subjects having a normal serum creatinine, urea nitrogen or nonprotein nitrogen level. Individuals with renal function ranging from normal to a 75 per cent reduction shoud be treated in the same manner. For patients in frank uremia, but with glomerular filtration rates estimated to be between 10 to 30 ml. per minute as determined by creatinine clearance, a maintenance dose given once every one or two days should suffice. For the very severely uremic patient who is anuric or has a creatinine clearance of less than 10 ml. per minute, a maintenance dose every two to four days would seem reasonable.[3] A periodic check on the serum

[3] A more precise basis for the dosage schedule in patients with different degrees of renal functional impairment can be obtained from the data presented in Figure 1 and in the following formula: Log half-life tetracycline = 0.55 ± 0.16 (log Ccr) + log 82 hrs.

level would still be advisable. For the patient with acute tubular necrosis undergoing recovery, a sequence of improving degrees of renal function must be anticipated and dosage schedules changed accordingly. The limited observations with chlortetracycline suggest that similar dosage regimens would be required to sustain levels of this drug in patients with either normal or impaired renal function.

Although the therapeutic index of the tetracyclines during ordinary dosage schedules may be considered rather high, evidence has slowly accumulated that large quantities of the tetracycline drugs in the body are undesirable. Gabuzda and co-workers (5) studied the effect of chlortetracycline at a dose of 3 Gm. per day in seven undernourished subjects and observed the development of a negative nitrogen balance, a presumably related rise in blood nonprotein nitrogen, increased urinary excretion of riboflavin in all subjects and of tryptophan, histidine and threonine in some of them. These changes could not be corrected by the administration of riboflavin and were not observed with oral administration of bacitracin or polymyxin B. Similar observations were made with oxytetracycline by the same authors and with tetracycline by Faloon, Noll and Prior (6). Womack and co-workers (36) noted an elevation in the blood urea nitrogen in patients during oxytetracycline therapy, and Bateman and associates, (12) using oxytetracycline in patients in the terminal stages of carcinoma who already had elevated blood urea nitrogen values, observed a further marked rise during treatment and this was associated with severe nausea and vomiting. It is difficult, however, to dissociate the effect of drug from the complications of the underlying disease in these cases.

Farhat, Schelhart and Musselman (37) gave rabbits large doses of tetracycline and observed rising nonprotein nitrogen levels, anorexia, weight loss, lethargy, convulsions and respiratory failure starting at serum levels of 20 μg. per ml.; oxytetracycline concentrations of 80 to 320 μg. per ml. of serum were found to be lethal. We have observed serum levels up to 32 μg. per ml. in uremic patients after relatively short courses of tetracycline, and Wood and co-workers (38) reported a serum concentration of 80 μg. per ml. in an oliguric patient on the fourth day of therapy. Since in the rabbit experiments referred to above (37), the signs of presumed drug toxicity and uremia were very similar, the potential toxic effect of the drug could easily be overlooked in the patient who is already severely ill. For this reason, it would seem wise to attempt to achieve and maintain in these patients serum levels that are not much in excess of those which are therapeutically effective. Since the metabolic effect of *in vivo* inactivated chlortetracycline is unknown, it cannot be assumed that this drug would be less toxic than tetracycline in uremic patients.

SUMMARY

The persistence of tetracycline and chlortetracycline in serum has been studied in patients with varying degrees of renal impairment up to almost total anuria. The half-life of intravenously administered tetracycline in individuals with normal renal function was about six to seven hours when measured by the twofold dilution method. With decreasing renal functional capacity as measured by the endogenous creatinine clearance, the half-life was prolonged and markedly so when the creatinine clearance fell below 30 ml. per minute; in the anuric patients it was as long as four to five days.

Tetracycline is cleared to a lesser extent than is creatinine or urea by hemodialysis across the artificial kidney; this is presumed to be due to the binding of the antibiotic to the plasma proteins.

In contrast to the findings with tetracycline, the half-life of chlortetracycline in serum in patients with severe renal disease is only slightly elevated over normal values, and hemodialysis by the artificial kidney did not result in lowering of the serum levels of this drug. The reasons for concluding that this difference is due to the rapid *in vivo* inactivation of this antibiotic are discussed. The poor extraction of tetracycline and particularly of chlortetracycline, by the artificial kidney is interpreted as being the result of binding of these drugs to serum protein.

The implications of these data for the potential toxicity of these drugs in uremic patients are discussed and an empiric dosage schedule is offered for use in such patients.

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PERSISTENCE OF ANTIBIOTICS IN BLOOD OF PATIENTS WITH ACUTE RENAL FAILURE. II. CHLORAMPHENICOL AND ITS METABOLIC PRODUCTS IN THE BLOOD OF PATIENTS WITH SEVERE RENAL DISEASE OR HEPATIC CIRRHOSIS * †

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It is known that the kidney plays a major but variable role in the elimination of most of the antibiotics that are used systemically. Since these drugs are often employed in the prevention and treatment of infections in patients with severe renal disease, it becomes essential, both for the choice of agents and for their most effective use, to know how the different antibiotics are handled by the patient with impaired renal function. The present series of studies was therefore undertaken with a view to providing a basis for the rational choice of antibiotics and their dosage in such patients. The persistence of several other commonly used antibiotics in the blood of patients with anuria and severe renal impairment was described in other papers of this series (2, 3). This paper is concerned with the overall metabolism of chloramphenicol in similar patients. In addition, since the liver is also involved in the metabolism of chloramphenicol, studies in a few patients with severe hepatic cirrhosis are also included.

The structure of chloramphenicol and possible sites of chemical alterations as they occur in the body are shown in Figure 1. A summary of what is currently known about the metabolism of chloramphenicol in man is presented schematically in Figure 2. Following the adminstration of chloramphenicol by the oral or parenteral route, 75 to 90 per cent of the administered dose may be recovered in the urine of patients who have normal renal function (4, 5). From 5 to 15 per cent of the recovered drug is in the microbially active form; the remainder is composed largely of an inactive monoglucuronide conjugate and to a lesser extent products of hydrolysis (6). Aryl amine derivatives of chloramphenicol are not ordinarily detectable in the serum or urine of man. About 3 per cent of the administered dose of chloramphenicol is excreted into the bile (4, 7) but less than 1 per cent is recoverable in the feces, largely as aryl nitro and amine derivatives (4).

In sharp contrast to the situation in man, the principle pathway of excretion of chloramphenicol in the rat is by the biliary system (8). When the drug reaches the intestine of the rat, the glucuronide linkage is hydrolyzed by the action of the bacterial flora, regenerating chloramphenicol (9). The nitro group may also be reduced to an amine (9) which is partially reabsorbed and excreted into the urine (8). Up to 25 per cent of the administered dose may be recovered in the urine of rats as inactive aryl nitro and amine derivatives.

In the present study, the half-life of chloramphenicol in serum and the 24 hour urinary recovery of chloramphenicol and its metabolic products were determined following single intravenous injections of the drug. These values were used to compare the fate of chloramphenicol in patients with renal and hepatic disease with that in normal subjects.

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MATERIALS AND METHODS

Patients studied and drugs used. The patients in whom these studies were made include four who were anuric, 11 who had varying degrees of renal impairment but normal liver function, 11 with nutritional (mostly alcoholic) cirrhosis of the liver but without significantly reduced renal function and four normal subjects. The levels of chloramphenicol and its derivatives were determined in the serum of six additional anuric patients who had been receiving chloramphenicol therapeutically. For purposes of the present study, anuria is defined as the condition in which a uremic patient excretes less than 400 ml. of urine daily for three or more days. It was assumed that the creatinine clearance in anuric patients was less than 1 ml. per minute. The diagnosis of cirrhosis of the liver was based on clinical and laboratory findings and in many of the patients was confirmed by liver biopsy.

Chloramphenicol [1] was supplied for intravenous use in ampules containing 500 mg. dissolved in 2 ml. of a 50 per cent aqueous solution of N,N-dimethylacetamide. The contents of one vial was diluted to 150 ml. in 5 per cent aqueous solution of dextrose and injected intravenously over a period of 15 to 20 minutes. Venous blood for the assays was drawn before and two hours after the injection and at convenient intervals thereafter; at least five specimens were obtained following each injection.

Assays for chloramphenicol and its derivatives. The concentration of antimicrobially active chloramphenicol in the serum and urine was determined by a turbidimetric assay using a strain of *Shigella sonnei* (10). Most of the control sera from jaundiced patients, however, were found to inhibit this organism; such sera were, therefore, assayed by the same method using *Agrobacterium tumefaciens* which was not inhibited by control sera. The concentration of total nitro compounds *plus* amines was determined by the titanous chloride reduction procedure (11); the aryl amines were determined on the same filtrates by the method of Bratton and Marshall (12) and the concentration of nitro compounds was calculated by the difference. All results are expressed in terms of chloramphenicol equivalents.

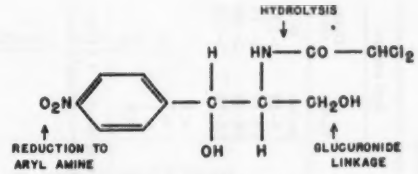

FIG. 1. SITES OF METABOLIC ALTERATIONS IN STRUCTURE OF CHLORAMPHENICOL

Identification of chloramphenicol and its products. Chloramphenicol glucuronide was identified in urine and in serum filtrates by paper chromatography using the descending procedure with butanol-phenol-pyridine solvent described elsewhere (6). The urine was applied directly to the paper strips; the serum was first deproteinized with five volumes of ethanol, centrifuged, and the supernatant concentrated by evaporation at room temperature. A known sample of chloramphenicol glucuronide (6) was used for comparison of R_f values; the presence of this compound in the unknown samples was confirmed by the addition of β-glucuronidase which resulted in the regeneration of microbially active chloramphenicol. After chromotography, the location of the aryl nitro compounds on the paper strips was established by reduction with titanous chloride, bromination, diazotization and coupling (6).

The following terms are therefore used in this paper to describe chloramphenicol and its metabolic derivatives: *1) Microbially active chloramphenicol,* as determined in the turbidimetric assays; *2) Aryl amine derivatives,* indicating the value determined by the Bratton-Marshall method; and *3) Total nitro compounds,* which includes the microbially active drug *plus* the inactive nitro derivatives.

Calculations of half-life and clearances. The serum

[1] Supplied by Parke, Davis and Company as Chloromycetin® solution.

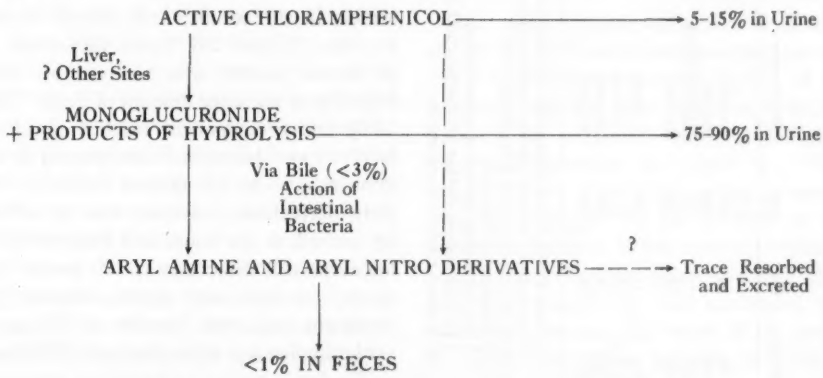

FIG. 2. METABOLISM OF CHLORAMPHENICOL IN NORMAL MAN

TABLE I

*Persistence of chloramphenicol and its derivatives in the serum of anuric patients and normal subjects after intravenous administration**

Patient	Sex	Age	Renal status†	BUN	Period observed	Urine output		Decay in serum		Half-life in serum		Aryl amines	
						Mean	Range	Active chloramphenicol	Total nitro compounds	Active chloramphenicol	Total nitro compounds	Range	Duration
		yrs.		*mg. %*	*hrs.*	*ml./da.*	*ml./da.*	*%/hr.*	*%/hr.*	*hrs.*	*hrs.*	*μg./ml.*	*hrs.*
Pe	M	43	Anuric	170	120	20	0–50	19.6	1.0	3.5	68.5	4–11	>120
Su	F	46	Anuric	160‡	10	350	350	16.0	§	4.3	§	12–17	> 10
Fi	M	47	Early diuretic	230	59	275	0–655	21.7	0.4	3.2	154.3	28–31	> 60
Sr	M	26	Anuric	200	22	0	0	‖	§	‖	§	13–16	> 22
Le	M	58	Normal	¶	22			20.8	15.9	3.3	4.4	<1	
La	M	63	Normal	¶	22			23.0	19.5	3.0	3.7	<1	
Ro	M	59	Normal	¶	22			23.9	15.2	2.9	4.6	<1	
Br	M	26	Normal	¶	22			43.3	15.3	1.6	4.5	<1	

* Dose was 0.5 Gm. except for Su who was given 1.0 Gm.; Fi had received 16 Gm. orally in eight days ending eight days before the I.V. test dose of 0.5 Gm.
† Etiology of anuria: severe hemolytic crisis (Pe); salmonelloses with diarrhea and shock (Su); acute glomerulonephritis (Fi); and acute hemorrhagic pancreatitis with mesenteric thrombosis (Sr).
‡ Nonprotein nitrogen.
§ No fall during period of observation.
‖ Patient received other antibiotics that interfered with the microbiological assay.
¶ Normal creatinine clearance.

half-life (T/2) was calculated by the method of least squares (13). The degree of renal impairment was estimated from the 24 hour endogenous creatinine clearance (Ccr) (14), a modification of the method of Bonsnes and Taussky (15) being used to determine the concentration of creatinine in serum and urine.

Dialysis by the Kolff-coil artificial kidney was performed for therapeutic purposes in three anuric patients, the extraction ratio (ER) of creatinine and chloramphenicol being determined as in the previous paper (3).

RESULTS

Anuric patients

Table I presents data on four anuric patients and four normal subjects who were given a dose of chloramphenicol intravenously. The half-life of the microbially active drug in the serum of anuric patients does not differ greatly from that observed in the the normal subjects. In contrast, the serum half-life of the nitro compounds is markedly prolonged in the anuric patients over that found in the normals. In addition, aryl amines which are usually < 5 μg. per ml. in serum of patients with normal renal function, were present in the anuric patients at levels ranging from 4 to 31 μg. per ml. and persisted for long periods. It is not known whether this is due to drug metabolites or to the presence of other substances.

Figure 3A presents the serum levels of the active and total nitro compounds as determined in the four normal subjects following an intravenous injection of chloramphenicol. The peak levels of either component at two hours after the intravenous injection did not exceed 8 μg. per ml. and at 24 hours little or none was demonstrable in the serum; aryl amines were not detectable. Figure 3B shows the serum levels in an anuric patient who received an intravenous injection of the same amount of drug. The microbially active component reached about the same levels at two hours and disappeared at about the same rate as in the normal subjects. The total nitro derivatives, however, rose to a level of 28 μg. per ml. at six hours and then declined slowly, but were still detectable at 120 hours. The level of aryl amines was slightly elevated prior to treatment and rose steadily to 11 μg. per ml. (chloramphenicol equivalent) at 120 hours.

The latter patient had received procaine penicil-

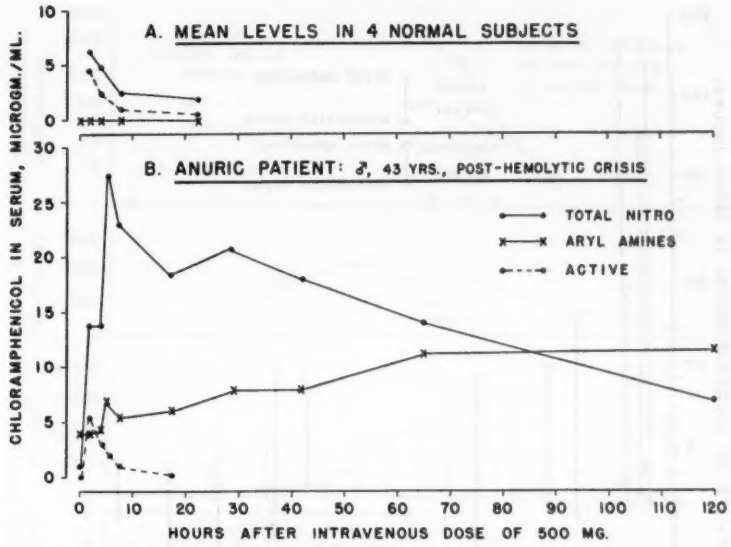
A. MEAN LEVELS IN 4 NORMAL SUBJECTS
B. ANURIC PATIENT: ♂, 43 YRS., POST-HEMOLYTIC CRISIS
TOTAL NITRO
ARYL AMINES
ACTIVE
CHLORAMPHENICOL IN SERUM, MICROGM./ML.
HOURS AFTER INTRAVENOUS DOSE OF 500 MG.

FIG. 3. MEAN LEVELS OF CHLORAMPHENICOL AND ITS METABOLIC PRODUCTS IN SERUM
A) Four patients with normal renal function, and B) an anuric patient after a single intravenous dose of 500 mg. of chloramphenicol.

lin for several days up to 18 days prior to this study; the possibility was therefore considered that this might have accounted for the finding of aryl amines in this patient's serum prior to the administration of the chloramphenicol. In order to obtain further data on this possibility and also to determine whether uremia *per se* could give rise to substances in the serum that react like aryl amines in the Bratton-Marshall method, sera were obtained from 12 uremic patients who had not received chloramphenicol. Levels of aryl amines, equivalent to 1 to 38 μg. of chloramphenicol per ml. were demonstrated in these sera. In most of these patients, however, there was a history of administration of some drug which gave a positive test for amines such as procaine (usually as procaine penicillin) or chlorothiazide. Previous administration of other aryl amines that are diazotized in this method, such as sulfonamides, acetazolamide and para-aminobenzoic acid, could not be excluded in all cases and many of these patients had minor surgical procedures during which procaine was injected. For these reasons, additional sera were obtained from seven uremic patients (nonprotein nitrogen values ranging from 63 to 165 mg. per 100 ml.) in whom previous administration of such substances could definitely be excluded. In most of these sera the level of aryl amines was less than 1.0 μg. per ml. and the highest value was 1.5 μg. per ml. It was therefore concluded that the finding of aryl amines in the blood of the uremic patients usually resulted from the administration of substances containing this moiety and their retention in the blood.

Patients with varying degrees of renal impairment

The mean half-life of active chloramphenicol and of the total nitro compounds was calculated for 11 patients with varying degrees of renal impairment. These data together with those of Table I are presented and correlated with the creatinine clearances in Figure 4. The serum half-life of the microbially active compound was not greatly altered by the severity of the renal disease as reflected in the level of creatinine clearance. On the other hand, the half-life of the total nitro compounds was prolonged when the creatinine clearance fell below 20 ml. per minute. In many of the uremic patients, the peak levels of nitro compounds in the serum was not reached

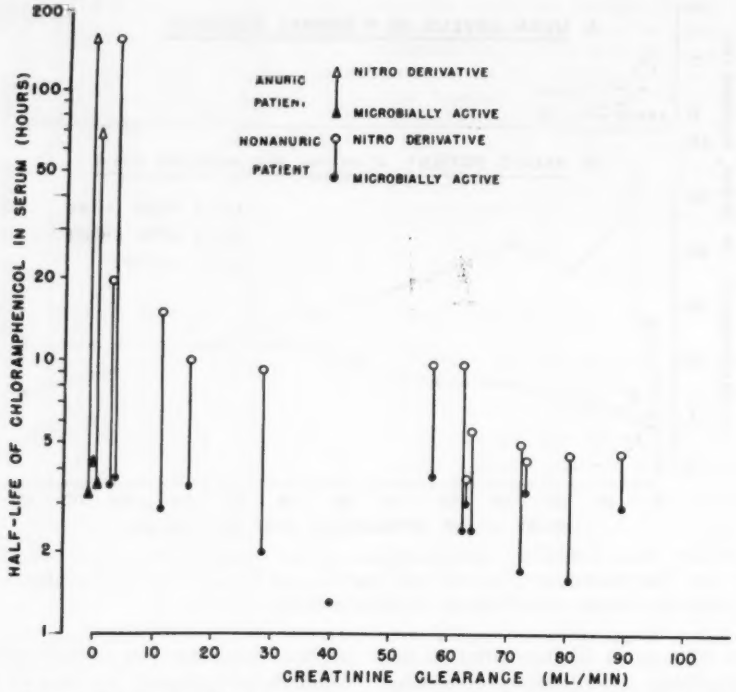

FIG. 4. HALF-LIFE OF MICROBIALLY ACTIVE CHLORAMPHENICOL AND OF TOTAL NITRO COMPOUNDS AS RELATED TO CREATININE CLEARANCE IN THE SERUM OF PATIENTS WITH NORMAL RENAL FUNCTION AND IN THOSE WITH VARYING DEGREES OF RENAL IMPAIRMENT

until five to 10 hours after administration of the drug; the reason for this was not determined, but it may be due to a difference in the relative volume distribution of chloramphenicol and its monoglucuronide. It has been shown that chloramphenicol is concentrated in tissues, whereas the glucuronide is water soluble and presumably is distributed in the extracellular water (4, 16).

Accumulation of chloramphenicol in the blood of patients on continuous therapy

The results presented above suggest that active chloramphenicol given in ordinary doses is inactivated nearly as rapidly in patients with severe renal failure as in normal individuals, but that in the former the total nitro compounds may ac-

TABLE II

Levels of chloramphenicol and its derivatives in anuric patients receiving chloramphenicol for therapeutic purposes

Patient	Age	Sex	Etiology of renal failure	Days of treatment	Total dose	Chloramphenicol in serum		
						Active	Aryl amine	Total nitro
	yrs.				*Gm.*	*μg./ml.*	*μg./ml.*	*μg./ml.*
Sa.	26	M	Acute hemorrhagic pancreatitis	2	4.0		13	50
St.	32	F	Premature separation of placenta	3	6.0	11.9		158
				10	20.0	10.2	47	611
K.	17	M	Ethylene glycol intoxication	7	8.5	23.0	40	333
W.	83	F	Postoperative	9	13.0	13.1		189
T.	68	M	Bladder perforation, shock	14	13.0	12.3	31	134
F.	47	M	Acute glomerulonephritis	8	16.0	<2.5	28	50

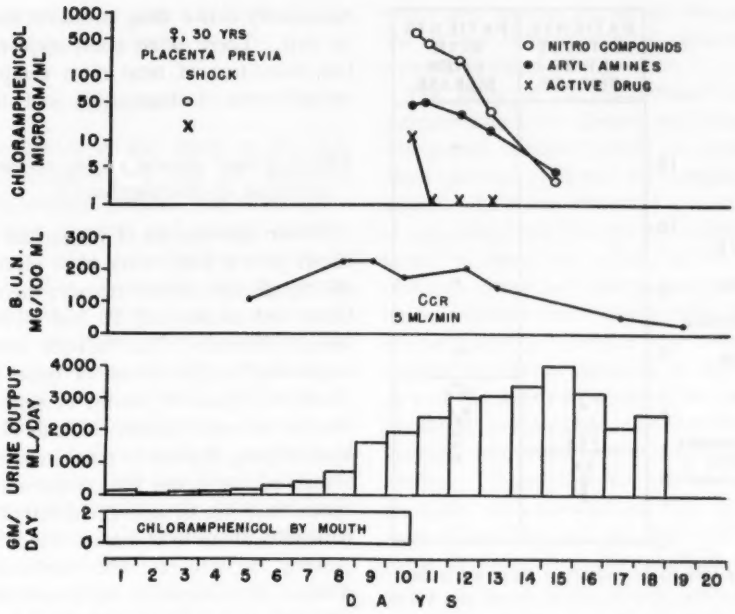

FIG. 5. LEVELS OF CHLORAMPHENICOL AND ITS METABOLIC PRODUCTS OBTAINED IN THE SERUM OF A PATIENT DURING THE DIURETIC PHASE FOLLOWING A PERIOD OF ANURIA IN THE COURSE OF WHICH SHE HAD BEEN TREATED WITH CHLORAMPHENICOL ORALLY

cumulate to very high levels. This is borne out by the data presented in Table II which shows the serum levels obtained during chloramphenicol therapy in six anuric patients. The highest level of active drug observed in this group was 23 μg. per ml. in a patient who had been treated for seven days, whereas the highest level of the total nitro compounds and aryl amine derivatives noted in Patient St. were 611 and 47 μg. per ml., respectively. The latter patient was in the diuretic phase of recovery and was no longer receiving the drug at the time the highest serum level was noted. The fall of chloramphenicol levels following cessation of therapy in this patient is illustrated in Figure 5; although the active drug was rapidly cleared from the serum, the total nitro compounds and the

TABLE III

Extraction of chloramphenicol and creatinine by the artificial kidney

Patient	Chloramphenicol*			Creatinine			
	Arterial	Venous	Extraction ratio (A)	Arterial	Venous	Extraction ratio (B)	A/B ×100
	μg./ml.	μg./ml.	%	mg. %	mg. %	%	%
Se (a)	44	37	16	22.0	15.2	31	52
(b)	39	34	13	18.9	13.4	29	44
Pe	7	4	43	22.0	5.0	77	56
Ta (a)	131 (22)	90 (17.6)	31 (20)	25.8	9.0	65	48 (31)
(b)	103 (18.2)	100 (15.0)	3 (18)	17.4	8.4	52	6 (35)
Mean			21 (19)			51 (59)	41 (33)

* Total nitro compounds were determined colormetrically. Data for the active chloramphenicol is shown in parentheses.

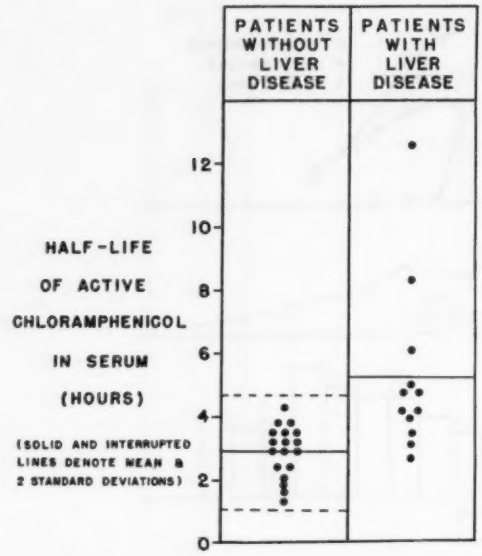

FIG. 6. HALF-LIFE OF MICROBIALLY ACTIVE CHLORAMPHENICOL IN THE SERUM OF PATIENTS WITH AND WITHOUT SEVERE LIVER DISEASE

aryl amines were removed more slowly. Paper chromatograhpy of the serum that showed the highest values for the total nitro compounds revealed that the major component had the same R_f as an authentic sample of chloramphenicol glucuronide.

Effect of hemodialysis with the artificial kidney on chloramphenicol levels

Three anuric patients were subjected to hemodialysis by the artificial kidney (Kolff-coil type) for therapeutic purposes. The results, exp. ssed as extraction ratio of the drug compared with that of creatinine, are presented in Table III. There was considerable variation in the extraction ratio of chloramphenicol and creatinine from one dialysis period to another. The comparative extraction of chloramphenicol and creatinine indicates that the nitro compounds were diffusible across the dialyzing membrane, but less so than creatinine. This may reflect the degree of binding of the nitro compounds to constituents of blood (16, 17). In general, the nitro compounds were cleared about half as well as creatinine and the microbially active drug probably about one-third as well. There is no ready explanation for the low extraction of total nitro compounds in the second series of observations in Patient Ta.

Effect of liver disease on the serum half-life and excretion of chloramphenicol

Eleven patients, all of whom had advanced cirrhosis of the liver, were each given 500 mg. of chloramphenicol intravenously; three of these patients had ascites and 10 had elevated levels of serum bilirubin. The half-life of active chloramphenicol in the serum of these 11 patients is shown in Figure 6; results of similar determinations in patients without cirrhosis of the liver, but with varying degrees of renal impairment are also shown in this figure for comparison. The mean serum half-life of active chloramphenicol in the patients without liver disease was 2.9 ± 0.9 hours. Among the patients with cirrhosis, there were marked differences in the serum half-life of the active drug, but at least three of the 12 observations showed a marked prolongation of the half-life over the normal values. The cirrhotic patient with the lowest serum half-life was the only one who was not jaundiced. However, the correlation between the serum half-life of active chloramphenicol and the total bilirubin level was imperfect; the correlation coefficient was $+0.54$, significant at the 5 per cent level, suggesting that factors in severe liver disease other than total bilirubin retention may be involved in the abnormal metabolism of chloramphenicol.

Table IV lists the total amounts of chloramphenical and its products recovered in the urine of six patients with hepatic cirrhosis during the 24 hours following intravenous administration of 500 mg. of chloramphenicol. Chromatographic analysis demonstrated high concentrations of glucuronide in these urines. Although three of these patients had clearances of creatinine that were below normal, the overall recovery of the drug was comparable to that reported for normal individuals (4, 5); this was true for active chloramphenicol as well as for the total nitro compounds. The defect in chloramphenicol metabolism in the patients having the longer serum half-life of active chloramphenicol therefore appears to be a reduced *rate* of conversion of active drug

to inactive products rather than a reduction in the total amount of drug converted.

DISCUSSION

The data presented in this study on the persistence of chloramphenicol and its metabolic products in the serum of patients with severe renal or hepatic disease confirm and extend observations made in previous investigations in normal man (4, 5, 7). The observation that the serum half-life of microbially active drug is not significantly prolonged in anuric patients indicates that the rate of removal of the drug by conversion to its metabolic products is virtually unaffected by renal disease. The prolonged serum half-life and the accumulation of the metabolic products in uremic patients imply that the rate of removal other than by renal mechanisms must be quite slow; in anuric patients it probably proceeds at less than 1 per cent per hour.

The prolongation of the serum half-life of chloramphenicol metabolites as the creatinine clearance falls is not as striking as that noted with tetracycline (3). This is presumably due to the fact that the chloramphenicol residues are cleared not only by glomerular filtration, but by active tubular secretion as well (4). Although there is generally a parallel decline in tubular function with falling glomerular filtration rates in uremic patients (18), this may not be adequately reflected in estimations of the glomerular filtration rate as determined by the creatinine clearance. The anuric patient, however, has a profound loss of tubular as well as glomerular functional capacity which may explain the abrupt change in the half-life of the chloramphenicol products in anuric patients as noted in Figure 4.

The study of chloramphenicol metabolism in patients with liver disease was hampered by the unexpected inhibitory action of serum from jaundiced patients on the test organism, *Shigella sonnei*. This was remedied, in part, by the use of *Agrobacterium tumefaciens* which was less affected by these sera. Some of the patients with cirrhosis converted chloramphenicol to its metabolic products more slowly than did normal or uremic patients. Despite this somewhat slower conversion rate as estimated by the decline in serum of the active drug, about the same total and fractional amounts of chloramphenicol and its products were recovered in the 24 hour urine collections. Since abundant chloramphenicol glucuronide was recovered in these urines, it would appear that the patients with cirrhosis are capable of conjugating chloramphenicol. Thus, the defect noted in these patients was in the rate of conversion rather than in the total amount converted. Similar results have been reported in patients with liver disease given a salicylamide load (19). Such findings emphasize the need for determining the rate of glucuronide synthesis rather than the total amount synthesized.

From the data presented, it would appear that one approach to achieving greater antibacterial activity of chloramphenicol in blood or urine would be to reduce the extent to which the drug is conjugated with glucuronic acid. N-acetyl-para-aminophenol (NAPA) has been reported to be conjugated with glucuronic acid to a much

TABLE IV

Urinary recovery of chloramphenicol and its products in patients with hepatic cirrhosis during 24 hours following an intravenous dose of 500 mg.

Patient	Total bilirubin	Ccr	Chloramphenicol in urine			Recovered in urine		
			Active	Aryl amine*	Total nitro	Active	Aryl amine	Total nitro
	mg. %	*ml./min.*	*mg.*	*mg.*	*mg.*	%	%	%
Hi	2.8	45.5	66	23	355	13.2	4.6	71.0
Bu	4.4	76.0	39	16	325	7.7	3.2	65.0
Da	0.3	82.0	78	25	433	15.7	5.0	86.6
Wa	3.5	80.7	62	30	325	12.2	6.0	65.0
St	1.8	103.1	71	32	417	14.3	6.4	83.4
Mr	3.9	62.0	41	28	333	8.2	5.6	66.7
Mean	2.8	74.9	60	26	365	11.9	5.1	73.0

* Normal urinary amines are included in these determinations.

greater extent than are salicylamide or acetylsalicylic acid (20). Corte and Johnson (21) also have reported that the administration of NAPA in doses of 250 mg. per Kg. to rabbits given together with cortisol or prednisolone resulted in a marked increase in plasma levels of 17-hydroxycorticoids which, like many other substances, are inactivated to a large extent by glucuronide formation (22). In a preliminary brief study carried out in two normal subjects, the administration of NAPA [2] did not appear to influence the metabolism of chloramphenicol to any significant degree. It is possible that more prolonged therapy or larger doses of either or both drugs, as compared with those used in this trial, may be necessary before an effect of NAPA can be demonstrated. Schmid and Hammaker (23), however, have recently shown that prolonged administration of NAPA may actually augment glucuronide production. In view of their findings it may be very difficult to overcome the glucuronidization mechanism by this type of competitive inhibition.

Chloramphenicol appears to be readily conjugated with glucuronic acid. Even where indirect bilirubin levels are high, suggesting poor conjugation of this substrate, large amounts of chloramphenicol glucuronide may be formed. It has been reported that the cat does not form glucuronates of phenolic substrates such as borneol, menthol, etc. (24). However, in other studies, oral administration of chloramphenicol to the cat has resulted in the excretion of large quantities of its glucuronide in the urine (25). Similiar experiments carried out in these laboratories with a strain of rats (26) that had been found deficient in the glucuronic acid conjugating mechanism for bilirubin, gave chromatographic evidence for the presence of chloramphenicol glucuronide in the urine following administration of chloramphenicol.[3] It is possible that the primary alcohol group as present in chloramphenicol is more readily conjugated than phenolic compounds or bilirubin.

The aryl amine derivatives of chloramphenicol are ordinarily present in very low concentrations in the serum or urine of patients under treatment (4). They appear gradually in the serum of uremic patients. The presence of aryl amines in the serum of uremic patients resulting from the administration of chloramphenicol invites comparisons with the situation in the rat in which large amounts of the aryl amine derivatives are excreted into the feces and urine. This has been shown to be the result of an active entero-hepato-renal circuit in the rat where nitro derivatives are excreted into the bile, reduced by the enteric flora to aryl amines and reabsorbed and excreted through the kidneys (8). Although in humans, only small amounts of chloramphenicol are excreted into the bile (4, 7), larger amounts may eventually get into the bile when renal clearances are markedly reduced. This may account for the gradual accumulation of aryl amine derivatives in the blood of uremic patients. It is also possible that reduction of the nitro group may occur in organs others than the gastrointestinal tract (4).

The accumulation of inactive chloramphenicol products in the blood of uremic patients raises the question of their potential toxicity for such patients. Chloramphenicol has been implicated as a causative factor in some cases of aplastic anemia (27–32). Since uremic patients are usually also anemic, further bone marrow depression that might result from chloramphenicol or its products may be difficult to detect. Of interest in this regard is a recent report of 15 patients observed during chloramphenicol administration in which five patients showed a reduced incorporation of iron into hemoglobin on the basis of which it was suggested that this drug may produce subclinical bone marrow depression (33). Although the possible role of renal impairment in these cases was not discussed, three of the five patients were listed as having renal disease, and two of the remaining 10 who showed no adverse effect from chloramphenicol are also listed as having some disease of the kidney.

Evidence has accumulated that at and soon after birth there is a deficient glucuronide forming mechanism (34–38). There is also a reduced tubular and glomerular functional capacity (39, 40) as compared with that of older children and adults. It would therefore be expected that both chloramphenicol and its products would be removed from the blood more slowly in those infants than in older children (41).

[2] Supplied as Atasol, Frank Horner, Ltd., Montreal, Canada.

[3] We are indebted to Dr. Rudi Schmid for providing the Gunn rats for this study.

Dialysis by the artificial kidney has been shown to reduce serum levels of both chloramphenicol and its nitro derivatives. Removal of large amounts of the drug by this method is hampered by the fact that the total nitro compounds are removed only about one-half as well as creatinine and even less of the active drug may be extracted.

Although the data on uremic patients indicates that normal dosage schedules must be used with chloramphenicol to maintain adequate therapeutic levels, the accumulation of inactive metabolic products introduces a new factor that must be considered. Although chloramphenicol glucuronide appears to be less toxic than the parent compound, the potential toxicity of the aryl amines is unknown. The hazards of administration of any drug to uremic patients must be recognized when the normal routes of elimination are unavailable. More serious results might be expected with compounds such as streptomycin which are normally excreted unchanged or with drugs whose metabolic products are more toxic than the original substance.

In patients with known hepatic impairment the overall metabolism of chloramphenicol appears to be essentially normal even though the rate of conjugation with glucuronic acid may be reduced. When renal function is adequate in cirrhotic patients, the prolongation of serum half-life does not appear to be great enough to justify any change from the usual dosage regimens.

SUMMARY

1. The serum half-life of active chloramphenicol is not significantly prolonged in anuric patients; on the other hand, the half-life of the metabolic products of chloramphenicol is markedly extended in patients with severe renal disease.

2. Small amounts of aryl amine derivatives may accumulate slowly in the blood of uremic patients receiving chloramphenicol.

3. Some patients with cirrhosis of the liver were found to have a prolongation of the serum half-life of active chloramphenicol. This appeared to be due to a slower rate of glucuronide conjugation in these patients.

4. Aryl nitro compounds of chloramphenicol were found to be extracted by the artificial kidney about one-half as well as creatinine.

5. These findings are discussed in relation to the potential toxicity of chloramphenicol products in uremic patients and in regard to dosage in patients with renal or hepatic disease.

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PERSISTENCE OF ANTIBIOTICS IN BLOOD OF PATIENTS WITH ACUTE RENAL FAILURE. III. PENICILLIN, STREPTOMYCIN, ERYTHROMYCIN AND KANAMYCIN * †

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In the preceding papers, one dealing with tetracycline and chlortetracycline (2) and the other with chloramphenicol (3), the circumstances under which these drugs would be expected to accumulate in the blood of patients with renal failure were indicated. Dosage regimens were outlined which took into account the limitations imposed by renal disease on the elimination of these drugs. The present report presents the results of studies on the persistence of four additional antibiotics; penicillin, streptomycin, erythromycin and kanamycin, following their administration to patients with severe renal failure. Since anuria may be considered to represent the maximum impairment of renal function as seen in man, emphasis was placed on the study of patients in this state in determining the maximum contribution of nonrenal factors toward the elimination or inactivation of these drugs under the physiological limitations of uremia. The findings should prove helpful in delineating the optimum dosage regimens for these drugs in patients with markedly impaired renal function.

MATERIALS AND METHODS

Definitions. Anuria is defined, for the purpose of this study, as the condition in which uremic patients excreted less than 400 ml. of urine per day for three or more days; patients who excreted somewhat more than 400 ml. per day are referred to as oliguric and when the urinary volume was in excess of 1,500 ml. per day they were considered to be in the diuretic phase.

The serum half-life (T/2) of each antibiotic was determined by the method of least squares (4).

Patients studied [1] *and antibiotics used. Penicillin* was given to 13 patients, including eight with severe renal failure and five without significant renal impairment; two of the anuric patients also had severe liver disease secondary to nutritional cirrhosis. The antibiotic was administered as a single injection of an aqueous solution of penicillin G and given as the potassium salt by the intramuscular route to three patients and intravenously as the sodium salt (dissolved in 20 ml. of distilled water or physiological saline solution and injected over a period of five to 10 minutes) to nine others. The remaining patient was studied after a course of procaine penicillin G. *Streptomycin* was given as a single intramuscular injection of 1.0 Gm., as the sulfate, to four severely uremic patients and to four control patients who had normal renal function. One uremic patient received 1.5 Gm. intramuscularly in a single injection and another uremic patient was given 3.5 Gm. over a three day period. *Erythromycin* was given as the glucoheptonate [2] to three uremic patients and to two controls; 0.5 Gm. of erythromycin base equivalent was dissolved in 10 ml. of distilled water and added to 100 ml. of 5 per cent dextrose in water solution and injected intravenously over a period of 15 to 20 minutes. *Kanamycin* [3] was given by the intramuscular route to two severely uremic patients and two controls in a dose of 1 Gm. as the sulfate.

Assay of antibiotics in serum.[4] Venous blood was drawn prior to the administration of each antibiotic and also one to two hours after completion of the injection and at convenient intervals thereafter. The blood was permitted to clot and the serum was separated and stored at $-20°$ C. until the time of the assay. Levels of penicillin in serum were determined by the twofold dilution method in brain heart infusion broth (Difco, pH approximately 7.4) using *Streptococcus 98* as the test organism, 0.5 ml. of a 10^{-4} dilution of the fully grown culture being added

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† A summary of some of these findings has appeared (1).

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[1] We are indebted to Dr. John P. Merrill for permission to study some of the patients on the Cardiorenal Service of the Peter Bent Brigham Hospital, and to Dr. Searle B. Rees for his cooperation in obtaining some of the specimens of blood from those patients.

[2] Kindly supplied by Eli Lilly and Company as Ilotycin Glucoheptonate®.

[3] Supplied by the Bristol Laboratories as Kantrex®.

[4] Clare Wilcox and Ann Najarian performed some of these assays.

to an equal volume of serum dilutions. Streptomycin was assayed in serum by the cup-plate method (5) using *B. subtilis* as the test organism. In the case of Patient Su, who had also received penicillin, sera were assayed by the twofold dilution method using as the test organism a strain of *Klebsiella pneumoniae* that was sensitive to 2 μg. per ml. of streptomycin and resistant to 64 μg. per ml. of penicillin. Comparable results were obtained when some of these sera were also assayed by the *B. subtilis* cup-plate method in the presence of 100 units per ml. of penicillinase.[5] Levels of erythromycin in serum were determined by both the twofold dilution method in broth and the agar-cup-plate method (5), employing *Sarcina lutea* as the test organism in each instance. Assay of erythromycin in the sera of one patient who had received a previous course of tetracycline therapy was performed by the twofold dilution method employing a freshly isolated strain of *Staphylococcus aureus* that was resistant to 62.5 μg. per ml. of tetracycline and sensitive to 0.12 μg. per ml. of erythromycin.

Assays of serum for kanamycin were done by the cup-plate method using *B. subtilis* (ATCC 6633) obtained from Bristol Laboratories and a modified streptomycin technique (5). Duplicate assays for kanamycin were done in this laboratory and by the Bristol Laboratories[6] and the results were in close agreement.

RESULTS

Penicillin

The data obtained in eight patients with severe renal disease and in five others who had normal renal function are presented in Table I; five of the former were anuric (as defined). The mean pencillin half-life in five patients with normal renal function who received the dose of 1,000,000 units of penicillin intravenously was 0.56 ± 0.17 hour; these values are similar to those observed by others (6). The serum decay rates expressed as per cent fall per hour ± 1 standard error are also presented in the table in order to demonstrate the inherent variability of the data.

The half-life of penicillin in the serum in Gi and Re during the anuric phase was 10.1 and seven and two-tenths hours, respectively. Patient Vo, who was totally anuric and in the terminal phase of hepatic cirrhosis, had marked ascites and jaundice; her serum bilirubin levels ranged from 18.0 to 20.7 mg. per cent during the study period. The penicillin half-life in this patient was 30.5 hours, the most prolonged observed in this group, even longer than that of Patient Fi (21.2 hours) who had received procaine penicillin and was totally anuric. Patient Do also had severe liver failure and was in the anuric category but was excreting about 100 ml. of urine per day. The serum half-life of penicillin in this patient was 16.4 hours which is significantly longer than in the anuric patients without severe liver disease after a dose of aqueous penicillin G.

Three patients (Gi, Si and Ma) were studied during the oliguric or early diuretic phases of their disease. At these times, the half-life of penicillin in the the serum of these patients was less than in the anurics, ranging from four to 10.6 hours. Three patients (Gi, Re and Su) were studied during the diuretic phase when the penicillin half-life ranged from three and nine-tenths to six and one-half hours. One individual who was followed through to early recovery (Gi) still demonstrated an abnormal disappearance curve with a half-life of six and three-tenths hours one month after onset of the anuria. Patient Su, who subsequently died of acute tubular necrosis and severe uremia, had a mean penicillin half-life of only three and one-half hours at the time of the last observations one month after the onset of anuria and shortly before death.

It can be seen from these data that Patients Vo and Do, who had severe hepatic impairment as well as anuria, differ markedly from the other patients in their inability to clear aqueous penicillin from their serum. They had the longest half-life and slowest rate of decay of penicillin in their serum.[7]

Streptomycin

Patient Su, who was studied while receiving penicillin, also was given 3.5 Gm. of streptomycin intramuscularly over a three day period shortly after completion of the first penicillin study. During the subsequent 20 days after the last dose of streptomycin, a number of specimens of serum were obtained and stored and all of them were subsequently assayed together for both penicillin and streptomycin. The results are presented in Figure 1. During these 20 days, the patient was

[5] Supplied by Schenlabs Pharmaceutical, Inc., New York, N. Y., as Neutrapen®.

[6] Courtesy of Dr. Kenneth Hubel.

[7] The only similarly prolonged half-life was in Patient Fi, but this patient received procaine penicillin G intramuscularly and the prolonged half-life is, in part, the result of the repository effect.

TABLE I

Persistence of penicillin G in serum of patients with severe renal disease, including two who also had severe liver disease

Patient	Renal status	Sex	Age	Precipitating factors in renal failure	Urine output		Period of study	BUN	Dose and route of penicillin*	Rate of decay of penicillin in serum	Half-life of penicillin in serum
					Mean	Range					
			yrs.		*ml./da.*	*ml./da.*	*days*	*mg. %*	*units*	*%/hr. ± S. E.*	*hrs.*
Gi	Anuric	F	25	Postpartum hemorrhage; shock	130	0–180	4	145	10^6 I.V.	6.8 ± 0.6	10.1
	Oliguric				403	150–690	3	105	10^6 I.V.	6.6 ± 0.5	10.6
	Diuretic				2,012	1,120–2,900	2	125	10^6 I.V.	17.8 ± 3.6	3.9
	Early recovery				880	600–1,230	3	85	10^6 I.V.	11.1 ± 1.5	6.3
Re	Anuric	M	79	Acute glomerulonephritis	295	30–400	4	210	10^6 I.V.	9.6 ± 0.8	7.2
	Diuretic				1,570	1,000–2,140	2½	240	10^6 I.V.	10.7 ± 0.3	6.5
Na	Early diuretic	F	43	Mismatched transfusion	450	450	2	60–71	10^6 I.M.	7.0 ± 1.0	9.8
Su	Early diuretic	M	38	Postoperative; shock	1,168	1,060–1,245	2	225–250	10^6 I.V.	15.8 ± 2.9	4.4
	Diuretic				1,890	1,795–1,950	2	230–270	10^6 I.V.	10.8 ± 2.0	6.4
	Severe failure†				600	600	1½	320–350	10^6 I.V.	19.8 ± 2.6	3.5
Ma	Oliguric	M	35	Subdural hematoma; shock	475	120–1,025	2	25–60	10^6 I.V.	17.5 ± 2.5	4.0
Fi	Anuric	F	38	Placenta previa‡	0	0	5	75–210	2.4×10^6 I.M.*	3.3 ± 0.3	21.2
Vo	Anuric	F	38	Hepatic cirrhosis§	0	0	5	70‖	10^6 I.M.	2.3 ± 0.8	30.5
Do	Anuric	F	41	Hepatic cirrhosis**	100	90–120	4	100¶	10^6 I.M.	4.2 ± 0.1	16.4
Five patients with normal renal function							1		10^6 I.V.	133. ± 39.	0.56 ± 0.17

* Aqueous sodium or potassium penicillin G, except in Fi, who received procaine penicillin G; I.V. = intravenous; I.M. = intramuscular.
† Creatinine clearance 27 ml. per minute; patient died shortly after this study.
‡ Bilateral cortical necrosis of the kidney found at autopsy.
§ Bilirubin 18 to 20.7 mg. per cent; died of liver failure.
‖ Value for NPN; creatinine = 8 mg. per cent.
¶ Nonprotein nitrogen.
** Bilirubin 15.9 to 36.5 mg. per cent; died of liver failure.

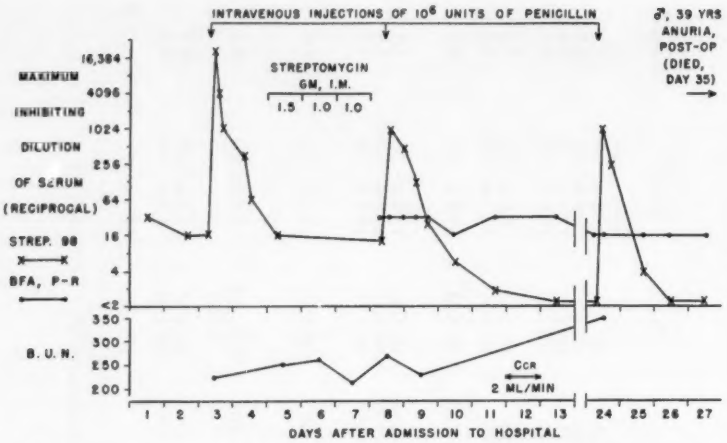

FIG. 1. PENICILLIN AND STREPTOMYCIN ACTIVITY IN SERUM OF AN ANURIC PATIENT AFTER INJECTIONS OF THESE ANTIBIOTICS

Activity of penicillin was determined by assay with *Streptococcus 98* that was moderately resistant to streptomycin. Activity of streptomycin was assayed with a penicillin resistant strain of *Klebsiella pneumoniae* (BFA-PR).

not anuric but his creatinine clearance was only 2 ml. per minute. The very slow fall in levels of streptomycin in the serum is quite striking particularly when compared to the decay rate of penicillin. The data presented in this figure are based on twofold dilution assays, but assays repeated with the cup-plate method using *B. subtilis* and penicillinase gave similar results.

Data in five other severely uremic patients and in four normal subjects all of whom received streptomycin are presented in Table II. It is evident that this drug was retained for long periods in the uremic patients The longest half-life observed was in Patient Go when she was anuric. The variations in the half-life noted in the three patients who were studied during the diuretic phase reflect the rate of recovery in these individuals.

Erythromycin

Table III shows data obtained in three patients with acute renal failure and in two patients with normal renal function, all of whom were given an intravenous dose of 500 mg. of erythromycin as the glucoheptonate. The half-life of erythromycin in the serum was one and two-tenths and one and six-tenths hours in the two control patients and ranged from four to five and eight-tenths hours in the anuric patients, including the one who was studied in the diuretic stage. These data suggest that nonrenal factors also play an important role in the clearance of this drug. Of interest is the finding that the half-life of erythromycin in the serum in Re, the patient with acute toxic hepatitis due to carbon tetrachloride, was not more prolonged than that observed in patients with anuria in whom liver function was apparently not impaired. The serum bilirubin level in this patient was 4.1 mg. per cent shortly before the erythromycin injection.

Kanamycin

Kanamycin levels in the serum were determined after a single intramuscular injection of 1 Gm. in two severely uremic patients and in two patients without renal functional impairment. The results are presented in Figure 2. The serum half-life in the controls was about four hours and it was greatly prolonged in the uremic patients. The half-life of kanamycin in the uremic patients was not calculated because of the wide fluctuations in the serum levels possibly due to shifts of water from various body compartments (both patients were edematous and receiving intravenous fluids) and possibly also because of irregular absorption of the drug from the intramuscular in-

TABLE II

Persistence of streptomycin in serum of patients with severe renal disease*

Patient	Renal status	Sex	Age	Factors precipitating renal failure	Urine output		Period of study	BUN	Rate of decay of streptomycin in serum	Half-life of streptomycin in serum
					Mean	Range				
			yrs.		*ml./da.*	*ml./da.*	*days*	*mg. %*	*%/hr. ± S. E.*	*hrs.*
Go	Anuric	F	63	Nephrectomy for thrombosis	210	100–340	4	100–147	0.6 ± 0.2	110.7
	Early diuretic			of renal artery	710	500–900	5	146–147	1.3 ± 0.2	51.8
Ro	Anuric	M	36	Acute glomerulonephritis	50	0–200	5	113–205	1.3 ± 0.2	52.1
Ke	Early diuretic	F	35	Posteclampsia	1,400	1,200–1,600	6	195–210	0.9 ± 0.2	75.0
Se	Diuretic	M	77	Transfusion reaction	2,700	930–5,600	4	138	2.5 ± 0.1	27.8
Th	Severe uremia	M	20	Acute glomerulonephritis	750	500–1,500	7	93–97	1.3 ± 0.2	52.8
Four normal men							1		29.5 ± 1.8	2.4

* All patients received 1.0 Gm. of streptomycin as the sulfate intramuscularly, except Ke, who received 1.5 Gm.

TABLE III

Persistence of erythromycin in serum of three patients with acute renal failure and in two normal subjects after an intravenous dose of 500 mg.

Patient	Renal status	Sex	Age	Factors precipitating renal failure	Urine output		Period of study	BUN	Rate of decay of erythromycin in serum	Half-life of erythromycin in serum
					Mean	Range				
			yrs.		*ml./da.*	*ml./da.*	*days*	*mg. %*	*%/hr. ± S. E.*	*hrs.*
Re	Anuric	F	46	Carbon tetrachloride	78	10–45	1	58–59	13.3 ± 0.7	5.2
	Anuric			poisoning*	120	100–130	1	93	14.4 ± 1.8	4.8
Ca	Anuric	M	29	Crush injury; shock	278	70–135	3	252	12.0 ± 1.2	5.8
Bo	Early diuretic	M	56	Penicillin reaction; shock	900	200–1,590	2	305	17.3 ± 0	4.0
	Diuretic				5,200	3,600–6,900	2	40	13.6 ± 5.0	5.1
Su	Normal	M	15				1		56. ± 5.	1.2
Ts	Normal	M	70				1		43. ± 8.	1.6

* Serum glutamic oxalacetic transaminase, > 3,000 units; bilirubin, 4.1 mg. per cent.

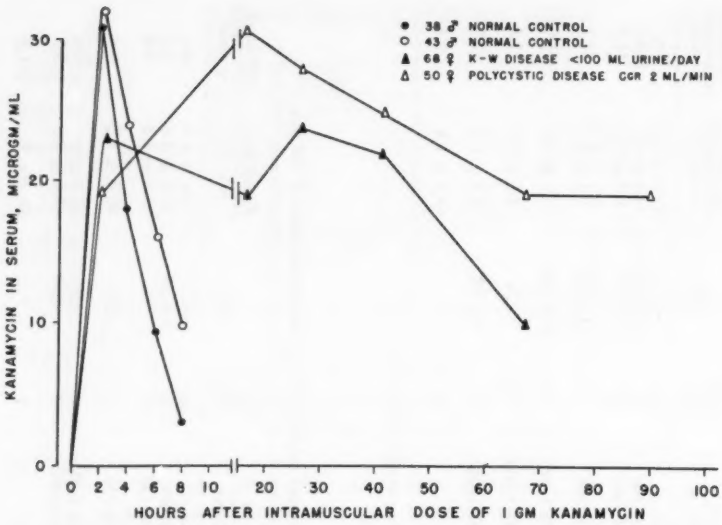
● 38 ♂ NORMAL CONTROL
○ 43 ♂ NORMAL CONTROL
▲ 66 ♀ K-W DISEASE <100 ML URINE/DAY
△ 50 ♀ POLYCYSTIC DISEASE CCR 2 ML/MIN
KANAMYCIN IN SERUM, MICROGM/ML
30
20
10
0
0 2 4 6 8 10 20 30 40 50 60 70 80 90 100
HOURS AFTER INTRAMUSCULAR DOSE OF 1 GM KANAMYCIN

FIG. 2. LEVELS OF KANAMYCIN IN SERUM AFTER INTRAMUSCULAR INJECTION OF 1.0 GM. IN TWO UREMIC PATIENTS AND IN TWO SUBJECTS WITH NORMAL RENAL FUNCTION

jection. It will be noted from this figure that rather high levels were still present in the serum three days after the injection of a single dose.

DISCUSSION

Penicillin

The clearance of penicillin in normal individuals is very rapid and is a combined function of glomerular filtration and active tubular transport (7). The renal removal of the drug is so efficient that other clearance mechanisms cannot ordinarily be considered of any consequence. In 1944, Humphrey (8) reported observations on two anuric women who had been given a single injection of aqueous penicillin G. He was unable to recover the drug in the small amount of urine excreted, but demonstrated penicillin activity in the serum for up to five days in each of these patients. Barnett, McNamara, Shultz and Tompsett (9) attributed the slower clearance of penicillin in premature infants to their limited renal function. In their studies, the ratio of the clearance of penicillin to that of inulin was 2.1 in prematures as compared with 4.4 in older children. Studies by Huang and High (10) in children of various ages given penicillin also demonstrated that premature and normal full term infants cleared penicillin much less rapidly than older infants and children. The renal clearance of penicillin by the aged is also decreased, paralleling decreases in renal function with age (11–13).

Beyer, Boger and co-workers (14) demonstrated that caronamide reduced the renal clearance of penicillin to approximately the glomerular filtration rate in normal subjects. Their data did not appear to indicate the presence of mechanisms of clearance of the penicillin other than renal excretion. However, in the experiments of Rowlands, Rowley and Stewart (15) who used radioactive sulfur-labeled penicillin in cats, only about 36 to 66 per cent of the administered dose was recovered in the urine as the active drug; the remainder consisted of unknown degradation products.

Studies by Eagle (16) on the *in vitro* inactivation of penicillins indicated that penicillin G is inactivated by human serum at a rate of 10 per cent per hour when incubated at 37° C. This corresponds to a half-life of about seven hours and is considerably more rapid than the value of 60 hours reported for inactivation in aqueous solutions of penicillin at pH 7.5 at 37° C. (17).

Anderson and Broderson (18) studied the velocity constants of the clearance of penicillin

from the plasma of dogs; following bilateral nephrectomy, the mean velocity constant fell from a normal value of 1.70 to 0.41 "reciprocal hours." The rate then fell to zero in animals which had been completely eviscerated or had combined nephrectomy and hepatectomy. These results would indicate that the liver rather than the plasma accounts for the major nonrenal clearance of the drug in the dog. The same would appear to be true in man, since in Patients Vo and Do, who had severe disease of both the liver and the kidneys, the half-life of aqueous penicillin G was the most prolonged, namely 30.5 and 16.4 hours, respectively. The half-life in the two anuric patients without hepatic disease was seven and two-tenths and 10.1 hours, which is somewhat greater than that found by Eagle in his *in vitro* studies with human serum (16). Since both of these patients were still excreting some urine, these values must be considered to be even less than would be expected in a totally anuric subject.

Thus it would appear that there is only a fair correlation between the *in vitro* serum studies and the *in vivo* findings. A possible explanation for these seemingly divergent data may lie in the fact that only a part of the total body penicillin store is in contact with plasma at any time. Spitzy and Hitzenberger (19) reported that the relative distribution volume of penicillin G is 0.56 ± 0.17 ml. per Gm. body weight in humans, indicating that the drug must be distributed in a relative volume that is about 10 times greater than the intravascular compartment. Also, protective substances may be present in the body which are not detected in the *in vitro* experiments.

From the therapeutic point of view Bull (20) indicated that a single injection of penicillin would suffice for prolonged periods in the anuric patient. The data presented in the present study would indicate that, in the anuric patient, a single injection would not be sufficient to maintain therapeutic levels for more than one or two days, if that long. Further injections would have to be given after a loading dose at intervals of four to 10 hours, depending upon the severity of the renal impairment. Also a large accumulation of penicillin cannot be expected to occur in the anuric patient given continuous therapy since the drug is still inactivated at a fairly rapid rate. Aqueous penicillin G may be expected to achieve the same prolongation of levels in the blood of anuric subjects as those achieved with procaine penicillin in the normal individual, but at higher serum concentrations after the same dose.

Streptomycin

Previous investigations have pointed out that in the presence of uremia streptomycin will accumulate in the blood if given continuously (21, 22). The data presented above indicate that a single dose given to a severely uremic patient may persist at reasonably high levels for many days. Several investigators (23–25) employing various methods of assay have reported that streptomycin is cleared primarily by glomerular filtration at rates varying from 30 to 70 ml. per minute. About 34 per cent of the drug is bound to plasma proteins (25) accounting for the fact that the clearance is less than that of the glomerular filtration rate. From 30 to 80 per cent of an intravenously administered dose has been recovered from the urine of normal individuals within the first 24 hours after injection (21–24, 26). Boxer and Jelinek (27) reported the serum half-life after intramuscular injection to be 2.72 ± 0.47 hours and similar values may be obtained by recalculation of the data reported by others (24, 26). The mean serum half-life of biologically active streptomycin determined in four normal men in the present study was two and four-tenths hours.

In view of these findings, one would expect the relation between streptomycin half-life in serum and the glomerular filtration rate to be somewhat similar although not identical to the findings reported for tetracycline (2). The half-life of streptomycin in anuric patients would appear from the present data to be similar to that of tetracycline. A conservative estimate of dosage increment necessary to maintain a reasonably high, but not excessive, serum level in a severely uremic patient can be suggested: following a loading dose of 1 to 2 Gm. of the drug, one-half the loading dose should probably be given every two to three days. The modification of dosage increments during the diuretic phase would not depend so much upon the amount of urine excreted as upon the recovery of renal function. For example, Patient Su had a very large urinary volume while maintaining fairly constant streptomycin levels

after a single dose, and Boxer, Jelinek and Edison (25) have shown that the clearance of streptomycin is independent of the rate of urine flow.

Erythromycin

The half-life of erythromycin in the serum of the anuric patients was clearly longer than that observed in the normal subjects. However, the maximum value observed in a uremic patient was only five and eight-tenths hours. This indicates that although the kidneys are of some importance in the clearance of this drug from the body, nonrenal mechanisms may be of greater importance. A considerable amount of erythromycin is excreted into the bile (28–30) and ordinarily only about 15 per cent of an adminstered dose may be recovered in the urine of normal patients. Data on the clearance of erythromycin is limited, but would imply that a considerable amount of plasma binding is present (31). The rapid decay of erythromycin in the anuric patient indicates that the artificial kidney cannot be expected to have any therapeutic importance in clearing the drug from the blood. The anuric patient should, therefore, be treated with nearly the same dose and at about the same time intervals as a normal patient.

Kanamycin

Data on the pharmacology and therapeutic uses of kanamycin have been presented in an extensive report (32). Chemically, this antibiotic has much in common with streptomycin, and like the latter it is a fairly stable drug cleared primarily by glomerular filtration (33) and recoverable in large amounts in the urine (34, 35). The data in the two severely uremic patients presented in this paper indicate that this drug is retained in the serum in patients with renal disease for considerable periods of time. Since the drug has been shown to have nephrotoxic and ototoxic potential it would seem wise to avoid using this drug in patients with severe renal disease and certainly to avoid overdosage when it is used. If it is found necessary to give this drug to anuric patients, they should not receive increments more frequently than every three or four days and these increments should be approximately one-half of the loading dose used.

Synthesis of data on antibiotics in renal disease

With the exception of penicillin and the metabolic products of chloramphenicol (chiefly the monoglucuronide) the antibiotics currently in use are cleared by the kidney by glomerular filtration. The rate at which each antibiotic is cleared is dependent upon the extent to which it is bound to plasma proteins. Some of the antibiotics are metabolized or excreted by other channels at a rate so rapid that the contribution of the renal clearance may be almost negligible (chloramphenicol and erythromycin). The serum half-life of each antibiotic reflects the rate of both renal and nonrenal clearance mechanisms. Figure 3 depicts a hypothetical representation of the relation between renal function and the half-life of antibiotics in serum. Two hypothetical drugs are considered: Drug A, which is completely stable and cleared only by renal mechanisms, and Drug B, which is cleared entirely by nonrenal mechanisms. An arbitrary half-life in serum for individuals with normal renal function is established at five hours for each drug in this model.

The following considerations were taken into account in constructing Curve A: *1*) The hypothetical substances, like inulin and mannitol, are not metabolized; *2*) the drug is removed entirely by renal mechanisms; *3*) clearance is independent of the rate of urine flow; *4*) serum half-life (T/2) is determined after distribution and equilibration in body compartments has occurred; and *5*) the relative volume distribution of the substance is independent of renal functional capacity. Then following the analysis presented by Newman, Bordley and Winternitz (36) in a study of the interrelation of the clearance of mannitol by the kidneys to the fall in plasma after a single intravenous injection we find that:

$$C = V_e S, \qquad 1)$$

where C is the rate of renal clearance (here expressed as the per cent of normal renal function including all modes of excretion), V_e is the relative volume distribution of the substance, and S is the serum decay rate. Since:

$$T/2 = \frac{\log 2}{S}, \qquad 2)$$

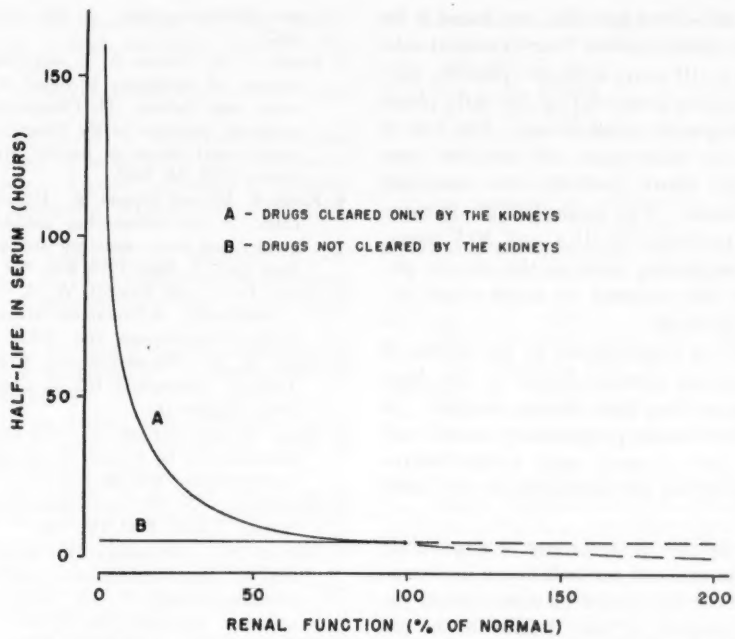

FIG. 3. REPRESENTATION OF HYPOTHETICAL RELATIONSHIP BETWEEN HALF-LIFE OF DRUGS IN SERUM AND RENAL FUNCTION FOR TWO CLASSES OF DRUGS

then

$$S = \frac{\log 2}{T/2}, \qquad 3)$$

and substituting in Equation 1 we find that

$$C = \frac{V_e (\log 2)}{T/2}. \qquad 4)$$

The reciprocal relation between renal function and half-life in serum of the injected substance noted in Equation 4 permits construction of Curve A in Figure 3. This shows that a marked prolongation of serum half-life does not occur until renal function has fallen below 20 to 30 per cent of normal, at which point the blood urea nitrogen and creatinine will also rise in the blood. Curve B, on the other hand, reflects the fact that the serum half-life of Drug B is unaffected by the extent of renal dysfunction or hyperfunction.

Although drugs such as tetracycline, streptomycin, kanamycin and the chloramphenicol metabolites have characteristics in common with Drug A, they are excreted in part by nonrenal mechanisms as well. Although active chloramphenicol and to a lesser extent erythromycin and penicillin resemble Drug B in many respects, all of these drugs are excreted into the urine to varying extents. Thus, as was found in the studies of tetracycline (2) and chloramphenicol (3), each drug tends to resemble either Model A or B, but is identical with neither. The anuric patient permits an estimation of the half-life of the agent in serum which approximates that which may be expected in the absence of any renal excretory function, whereas the normal patient fixes the other extreme of the curve. By this approach the limitations of renal disease upon the persistence of a drug may be established.

These principles may also be useful in considering the effect of renal disease on the persistence of drugs other than antibiotics in the blood of patients with renal disease.

SUMMARY

The effect of severe renal disease on the persistence of penicillin, streptomycin, erythromycin and kanamycin in the blood has been studied by determining the rate of their decay in the serum.

The serum half-life of penicillin was found to be prolonged from about one-half hour in normal subjects to seven to 10 hours in anuric patients, falling to four to seven hours during the early phase of recovery from acute renal failure. The role of the liver in the inactivation of penicillin was studied in two anuric patients who also had severe liver disease. The serum half-life in these patients was prolonged to 16.4 and 30.5 hours, respectively, suggesting that in the uremic patient, the liver may account for much of the degradation of the drug.

The half-life of streptomycin in the serum of anuric patients was found to be two to five days, falling to one to two days during diuresis. In one patient who became progressively uremic and subsequently died of acute renal tubular necrosis, streptomycin was still detectable 20 days after the last dose.[8]

The serum half-life of erythromycin was slightly prolonged from one and one-half hours in normal subjects to about five hours in three anuric patients. The presence of liver disease in one patient did not appear to prolong the serum half-life of erythromycin.

The serum half-life of kanamycin in two normal subjects was about four hours. Elevated levels of kanamycin were detectable in the blood of two severely uremic patients as long as four to five days following a single injection of 1.0 Gm.

A theoretical analysis of the effect of changing renal function on the serum half-life of antibiotics is presented; this may be applicable to other drugs as well.

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TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. III. A METHOD FOR SUBFRACTIONATION OF RS-1 SOLIDS *

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The total nondialyzable solids (TNDS) of normal human urine have been found to have an approximate composition of 47 per cent protides, 16.6 per cent glucides, 9.7 per cent sialic acid, 6.2 per cent hexosamine, 3.3 per cent lipids, 12.2 per cent bound water and 8.5 per cent ash (1). The mean TNDS excretion in two series of determinations has been found to be 433 mg. and 505 mg. per 24 hours, with a value of 472 (S.D. ± 108) mg. per 24 hours for the combined series (1, 2). Methods have been described for separation of the TNDS into three reproducible fractions (2). Figure 1 is a flow sheet illustrating the technique and approximate percentage weight distribution of each fraction. Boundary electrophoretic studies, at pH 8.6, have demonstrated the presence of concentration gradients in the RS-1 fraction which have mobilities closely approximating each of the gradients of normal blood plasma under similar conditions (3). The Cohn Method 10 for fractionation of plasma proteins has been modified by Lever and co-workers to permit separation of small quantities of plasma proteins (4). The present report concerns the application of this technique to the fractionation of RS-1 solids from normal human urine.

MATERIALS AND METHODS

Subjects. Ten to 12 subjects (seven male and five female) submitted 24 hour urine specimens, which were pooled daily for rapid processing (2).

Reagents. The reagents were prepared from stock solutions at room temperature and cooled to − 5° C. immediately before use. The reagents A and A′ of Lever gave an initial precipitation medium of pH 5.8. Γ/2 of 0.04 and 19 per cent ethanol when added to the requisite amount of acid-citrate-dextrose (ACD) plasma. The aqueous solution of RS-1 urinary fraction was found to lack the buffering action of ACD plasma; hence, in order to achieve the above conditions for urinary RS-1, the reagents A and A′ of Lever and co-workers were combined to form Reagent I.

Reagent I. This reagent contained 250 ml. 95 per cent ethanol, 20 ml. of M sodium acetate, 1.75 ml. of M acetic acid and water to make 1,000 ml. When necessary, the pH of this solution was adjusted to pH 5.80 ± 0.05 by addition of approximately 0.05 ml. of 10 M acetic acid.

Zinc reagent. This reagent contained 54.8 Gm. of zinc acetate dihydrate dissolved in 500 ml. of water, to which 200 ml. of 95 per cent ethanol was added and then sufficient water to make 1,000 ml. The final pH was 6.5 ± 0.1 when stored at 3° C.

Veronal buffer. This was prepared by dissolving 11.188 Gm. of analytical grade 5,5-diethylbarbituric acid and 82.4 Gm. of the sodium salt of this acid in 3,900 ml. of CO_2-free warm distilled water with final adjustment to a volume of 4,000 ml. at 20° C. This buffer had a specific conductivity of 3.11 (± 0.05) × 10^{-3} mho and a pH of 8.65 (± 0.03) at 0° C.

Apparatus. The fractionation was performed at − 5° C. in a cold bath of 50 gallon capacity equipped with a pump stirrer and with the temperature regulated to − 5.0 ± 0.5° C.[1]

All centrifugation was with a Servall superspeed refrigerated centrifuge set at − 5° C. with SS-1 35° angle rotor of radius 10.8 cm., providing a relative centrifugal force of 7,710 × G at 8,000 rpm.

Technique of fractionation. After much experimental variation in technique, the following procedure was adopted as the standard. Exactly 1.55 Gm. of pooled lyophilized RS-1 solids are suspended in 25 ml. of distilled water by gentle stirring for one minute and allowed to stand at 3° C. overnight. The resulting solution is dark brown in color, but clear. Centrifugation for 20 minutes at − 5° C. may give a sediment having a lyophilized dry weight approximating 2 to 4 per cent of the original RS-1 solids. The supernate is adjusted to exactly 25 ml. at − 5° C. by addition of distilled water and cooled to approximately 1° C.

Step 1. Five plastic centrifuge tubes of 40 ml. capacity are suspended in the bath and 2 ml. of precooled

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[1] American Instrument Company, Bath No. 4-86 G.

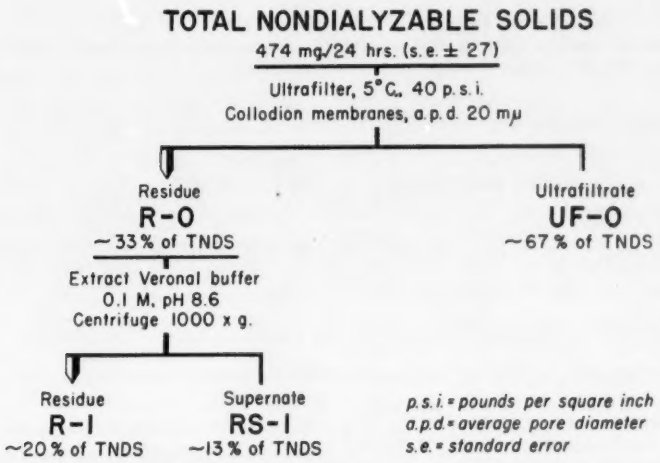

FIG. 1. FLOW SHEET ILLUSTRATING THE TECHNIQUE FOR FRACTIONATING URINARY TOTAL NONDIALYZABLE SOLIDS AND THE NORMAL PERCENTAGE WEIGHT DISTRIBUTION AMONG THE FRACTIONS AFTER LYOPHILIZATION

Reagent I added to each. Five ml. of precooled RS-1 solution is then added dropwise with constant stirring followed by dropwise addition of 18 ml. of Reagent I and continued stirring for 15 minutes. The tubes are then permitted to stand for 60 minutes.

The tubes are transferred directly into the precooled centrifuge and centrifuged for 30 minutes. The clear supernatant solution is removed, pooled and stored in the cold bath at − 5° C. The dark brown precipitate is returned to the cold bath and resuspended by gentle stirring for 15 minutes in 10 ml. of Reagent I. After standing for 15 minutes, the tubes are recentrifuged as above. The clear supernate is combined with the original to give a total volume of 175 ml. The residue, designated fraction RS-1A, is taken up in 2 ml. of veronal buffer with 1 ml. of buffer wash for each tube, giving a final volume of 15 ml. of solution. This material is then subjected to lyophilization after dialysis against distilled water at 3° C. for 72 hours. The lyophilized material is a white, fluffy, nonhygroscopic powder.

In the initial efforts to fractionate RS-1 solids by the above technique, the precipitation and filtration through sintered glass cups of 14μ maximal porosity was attempted.[2] This was unsatisfactory because of the high concentration of mucosubstances in the filtrate. These molecules adhered to the glass, thus blocking the filter pores to such an extent that filtration required 24 to 36 hours at a pressure of 38 cm. of mercury. There was a considerable loss of material by entrapment in the filters, even though much of this material appeared to be quite soluble in the precipitating medium, hence the need for centrifugation rather than filtration in Step 1.

The precipitate RS-1A is comparable to Cohn Fractions I, II and III. Since the quantity of solids in this fraction was relatively small and only two concentration gradients were seen by boundary electrophoresis, no further attempts to fractionate RS-1A were made.

Step 2. The supernate from Step 1 (which should total 175 ml. at this point) is measured, thoroughly mixed by gentle stirring, and 5.0 ml. of zinc reagent added dropwise for each 50 ml. of supernate, with constant stirring, at − 5° C. A greyish-brown flocculent precipitate forms immediately and stirring is continued for 15 minutes. The solution is allowed to stand in the bath for one hour, then transferred to six precooled 40 ml. plastic centrifuge tubes and centrifuged for 30 minutes. The clear and colorless supernate is removed and returned to the cold bath. A zinc acetate wash is prepared by addition of 8 ml. of zinc reagent to 100 ml. of Reagent I. The dark brown precipitate in each tube is resuspended in 15 ml. of precooled zinc acetate wash by gentle stirring and, after 15 minutes standing at − 5° C., the centrifugation is repeated. The clear supernate is combined with that above from the first centrifugation. This supernate, designated fraction RS-1C, is dialyzed against distilled water in preparation for lyophilization. The lyophilized product is a cream-colored, fluffy powder. This fraction is comparable to Cohn Fraction VI. The residues of each tube are taken up in 3 ml. of veronal buffer followed by a 2 ml. and 1 ml. wash with veronal buffer. The transfer funnel is washed with 1 ml. of veronal buffer to give a final volume of 37 ml. for the residue, which is completely soluble as a dark brown but clear solution. This fraction, designated fraction RS-1B, is comparable to Cohn Fractions IV and V. This material is dialyzed against distilled water and lyophilized for recovery of the solids. Lyophilization yields a tan, fluffy, nonhygroscopic powder.

Analytical methods. Analyses of the lyophilized solids for total nitrogen, hexose, "sialic acid," hexosamine,

[2] Corning Glass cups, size M.

TABLE I

Distribution of recovered solids in fractions of RS-1, expressed as per cent of lyophilized dry weights

Pool No.	12*	13	14	15	16	Human plasma†
Fraction						
RS-1A	4.3	1.4	0.9	6.4	3.8	32.8
RS-1B	75.4	65.8	68.4	68.5	72.3	65.6
RS-1C	20.3	32.7	30.8	25.1	23.8	2.2
Per cent of total recovered	48	57	79	78	52	
RS-1/R-O‡	0.33	0.51	0.38	0.31	0.31	

* Fractions from Pool 12 weighed after recovery from electrophoresis cell.
† Reference 4.
‡ Average for 7 pools, 0.36. R-O is the total nonultrafiltrable fraction of TNDS.

hexuronic acid (naphthoresorcinol method) and lipids were by previously described methods (1, 2). Sulfhydryl analyses were by the method of Ellman (5).

RESULTS

The electrophoretic analysis of RS-1 and of the three subfractions will be presented in a subsequent report.

Table I presents the percentage distribution by weight of the recovered solids in each fraction, the total recovery in terms of per cent of initial lyophilized RS-1 solids, and the ratio of RS-1 to R-O solids in each of five pools (representing 162 individual 24 hour urine volumes).

Table II presents some analytical results with fractions from three pools of RS-1. These pools were collected during the months of December through February for Pool 13, March through May for Pool 14 and June through July for Pool 15 as a part of the program for evaluation of seasonal variations in urinary TNDS excretion. After correction for loss during fractionation, the sums of the glucides, protides ($N \times 6.25$) and total lipids in each fraction account approximately

TABLE II

Analyses of starting samples of RS-1 and of their respective Cohn fractions, expressed as per cent of lyophilized dry weight

Fraction	Pool No.	Hexose*		"Sialic acid"	Hexosamine†	Hexuronic acid‡	N	Total lipids§	Total phospholipids	Total fatty acids plus unsaponifiable material‖	Total cholesterol
		Anthrone	Orcinol								
Total (RS-1)	13	11.7	11.3	5.2	8.9		10.6				
	15	13.0	9.1	2.6	7.5	0.21	10.6				
		(12.2)¶	(10.9)	(1.7)	(7.8)						
RS-1A	13	9.2	6.5	0.0	5.1		9.0				
	15	11.8	8.9	0.0	3.6		11.6				
		(11.3)	(9.9)	(0.0)	(3.8)						
RS-1B	13	7.9	5.1	0.0	5.7		11.5	9.84	0.38	4.70	0.67
	14	8.8	5.3	0.0	5.5		11.7	12.2	0.63	3.60	1.01
	15	9.8	6.4	4.7	5.0	0.18	11.8				
		(6.0)	(5.3)	(3.5)	(7.7)						
RS-1C	13	20.4	13.7	7.8	14.5		9.5	7.86	0.27	4.97	0.15
	14	24.2	18.8	6.8	15.4		7.3	14.77	0.29		0.43
	15	22.0	20.0	9.0	13.0		8.0				
		(24.1)	(20.0)	(7.3)	(13.5)						

* Standard was galactose and mannose, 1:1 by weight.
† Expressed as glucosamine HCl.
‡ Naphthoresorcinol method.
§ Chloroform extract. Insufficient A fraction was obtained to permit lipid analysis.
‖ Petroleum ether extract after saponification.
¶ Figures in parentheses are analyses obtained after redialysis and relyophilization of sample of Pool 15.

for their respective contributions to the composition and weight of RS-1 solids (2).

The isoelectric precipitation of the total RS-1B was complete within the pH range of 4.0 to 4.2. The RS-1C fraction behaved as a mixture of substances with isoelectric points varying between pH 2.2 and 3.5, although precipitation was never complete between the range 1.0 to 10.0

Both intact and hydrolyzed RS-1 solids contained less than 0.1 per cent sulfhydryl sulfur.

DISCUSSION

The system of fractionation used here requires critical control of pH, temperature, ionic strength and zinc concentrations. Deviations in technique to improve the rate of recovery are hence limited in scope. The precipitated mucosubstances tend to adhere to glass and even plastic surfaces so tenaciously that they cannot be removed by rinsing with the precipitating medium. In separate experiments these invisible films were recovered from the stirring rods and other apparatus by copious distilled water rinses which were subsequently lyophilized and weighed. The mechanical loss thus determined under optimum conditions was approximately 0.6 per cent of the 1.55 Gm. of starting solids.

The lyophilized RS-1 solids contained approximately 2 per cent of insoluble material which was lost in the initial centrifugation; this is not necessarily denatured protein, but may instead be traces of incompletely separated fraction R-1 (2). After lyophilization, approximately 4 per cent of RS-1 became dialyzable. It was found possible to increase the percentage of recovered solids to 89 per cent of the starting total by removing these substances in the initial step of this procedure. This was readily accomplished by redialysis of the aqueous suspension of RS-1 prior to the initial precipitation, but had the disadvantage that approximately half of the mucosubstances of RS-1B then appeared in the RS-1A precipitate.

This change of some component(s) of RS-1 from nondialyzable to dialyzable form (or other source of loss) apparently involves fractions RS-1B and RS-1C to an approximately equal extent, otherwise the proportions of these two fractions should vary with the per cent of total recovery. The proportional relationship among the three fractions was far more constant than the total recovery (Table I). Electrophoretic studies of RS-1 indicated the alpha components and the immobile component to be quantitatively the most variable (3). The former appear in both RS-1B and RS-1C, the latter in RS-1C only.

We have been unable to detect any seasonal variation in the amount or composition of RS-1 urinary solids.

The data of Table II demonstrate some chemical differences in the three fractions. The glucide content suggests a high concentration of mucosubstances in all fractions, being much the greatest relative to nitrogen content in RS-1C.

The detection of hexuronic acid only in the RS-1B solids is not evidence that it is absent from RS-1C. The hydrolytic conditions for the method used here are inadequate to release the hexuronic acid from (*e.g.*) chondroitin sulfuric acid (2), while interference prevents a reliable determination by the carbazole technique. Paper electrophoresis of these fractions, with commercial chondroitin sulfate as a control and color development with aqueous toluidine blue O, indicated the presence of metachromatic material with the mobility of chondroitin sulfate. The majority of this material appeared in RS-1B, although a trace was consistently present in RS-1C and none was detected in RS-1A. There was a suggestion that more than one metachromatic substance was present but trailing prevented a clear evaluation of the number of components.

The absence of sulfhydryl groups after hydrolysis of RS-1 solids is an indication of the absence of sulfur-containing amino acids. This, together with the low isoelectric points, the relatively high carbohydrate contents and the low albumin to "globulin" ratio is further evidence that materials having electrophoretic mobilities at pH 8.6 like serum globulins are chiefly mucosubstances and not primarily proteins which have escaped into the urine. The results of a number of other workers indicate that the situation is probably quite different with certain pathological urines [*e.g.*, (6)].

The results of Webb, Rose and Sehon (7, 8) indicate that the mucosubstances of RS-1 have molecular weights on the order of 10,000. This would be consistent with current ideas concerning the general relationship between molecular

size and selective appearance of materials in the urine. However, these workers are dealing with a combination of both the RS-1 and UF-O portions of the TNDS, which makes it difficult to compare their results with our own.

Total cholesterol in RS-1C was lower than in RS-1B. Values for "total lipids" and "fatty acids plus unsaponifiable matter" were of the same order of magnitude in the two subfractions and were also similar to, or only somewhat lower than, the values previously determined on the unfractionated material, RS-1 (2). However, in both subfractions figures for "total phospholipids" were considerably smaller than in RS-1 (one-half to one-eighth in RS-1B, and one-fourth to one-tenth in RS-1C), a finding which definitely suggests that phospholipids are to a large extent split and/or removed during the separation procedure.[3]

SUMMARY

The nonultrafiltrable, buffer-soluble fraction (fraction RS-1) of the total nondialyzable solids of normal human urine was subjected to the first steps of Cohn's Method 10 for the separation of plasma proteins. Three distinctly different and apparently reproducible fractions were obtained. It has not been possible to separate mucosubstances from proteins by this method. The low isoelectric points, high glucide content and absence of sulfhydryl groups suggest that the RS-1 solids are composed almost entirely of mucosubstances.

[3] The low phospholipid values and the sizable differences between the "total lipids" and the "total fatty acids plus unsaponifiable matter" indicate that the chloroform extracts of RS-1B and RS-1C included substances other than phospholipids, which were insoluble in petroleum ether or which, upon saponification, yielded a large proportion of products insoluble in this solvent. Some of these products might be hydroxyacids resulting from the oxidation of unsaturated fatty acids in the course of the manipulations. On the other hand, from the acidimetric titration of the petroleum ether extracts and from the weight of these extracts (corrected for the weight of cholesterol) the maximum possible values for the average molecular weight of fatty acids can be calculated. The figures so obtained (201 for RS-1B, Pool 13; 241 for RS-1B, Pool 14; and 232 for RS-1C, Pool 13) are distinctly below the corresponding values for the mixtures of fatty acids extracted from serum or tissues (276 to 284) and therefore suggest the presence of some organic acid soluble in petroleum ether, but with a rather short carbon chain and relatively nonvolatile.

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TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. IV. ELECTROPHORETIC PROPERTIES OF RS–1 SUBFRACTION *

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The nonultrafiltrable, veronal soluble components of the total nondialyzable solids (TNDS) of normal human urine have been designated as the RS-1 fraction (1). Separation of RS-1 into three subfractions by a modification of Cohn's Method 10 for fractionation of human plasma has been reported (2). The RS-1 and subfractions RS-1A (Cohn I, II and III), RS-1B (Cohn IV and V) and RS-1C (Cohn VI) have been subjected to boundary electrophoresis at pH 8.6 and 4.5. The comparison of concentration gradients appearing in these several fractions of urinary proteins and in normal human serum provides the subject matter of the present report.

MATERIALS AND METHODS

The human subjects and methods of collection of 24 hour urine specimens were the same as previously reported (1). The RS-1 fraction of 72 hour urine collections from each subject was recovered in the lyophilized state as previously described. Such a collection contained approximately 150 mg. of dry RS-1 solids.

Veronal buffer was prepared as previously described (2). The acetate buffer was prepared by dilution of 20 ml. of 2 N sodium acetate, 11.5 ml. of 3.5 M acetic acid and 32 ml. of 5 M sodium chloride to a volume of 2 L. with distilled water. This buffer at 1° C. had a conductivity of 5.12 ($\pm$ 0.05) $\times$ 10^{-3} mho and a pH of 4.5.

Electrophoresis. The moving boundary electrophoresis was done in a Beckman-Spinco model H electrophoresis-diffusion apparatus. The same standard long-channel (9.2 cm.) analytical cell of 11 ml. capacity was used in all experiments to minimize variation in calculations of mobility. A cadmium-mercury light source (General Electric No. H-100 F4) and the Philpot diagonal diaphragm-cylindrical lens optical system were used. Photographs were made on glass plates using a No. 29 Wratten contrast filter (dominant transmission 644 μ) for the Eastman high resolution spectroscopic plates (emulsion No. 514, 251), and a No. 15 Wratten contrast filter (dominant transmission 546 μ) for the Eastman high resolution metallographic plates.

Mobilities of the concentration gradients were calculated in the usual manner, with the exception that the distance of migration was measured from the advancing margin of the ascending protein buffer gradient and from the trailing margin of the descending protein-buffer gradient of the initial boundaries. This deviation was made necessary by the exceedingly high color of the urinary proteins, which obscured the exact center of the initial protein-buffer boundaries in some instances. The percentage composition of the various concentration gradients was determined by planimetry of photographic enlargements of the patterns after the method of Longsworth and MacInnes (3).

Although more than 50 specimens of RS-1 have been studied electrophoretically in this laboratory, the present report is limited to the following experiments conducted specifically for collection of these data.

Eleven subjects, eight male and three female, submitted three consecutive 24 hour urine specimens, from which the RS-1 was recovered as the lyophilized solids (1). These solids (approximately 150 mg. dry weight) were taken up in 12 ml. of veronal buffer, dialyzed against buffer overnight, and subjected to electrophoresis at 1° C. under a potential gradient of 5.6 volt/cm. for 14,400 seconds. The patterns were photographed at one hour intervals and the protein solution recovered from the cell.

The recovered protein was dialyzed against repeated changes of acetate buffer until pH and conductivity studies indicated equilibrium had been reached (three to five buffer changes in 72 to 96 hours). A small sediment was recovered on centrifugation of the acetate-equilibrated proteins. This precipitate was readily soluble in 4 ml. of veronal buffer, and after equilibrium dialysis these proteins were then subjected to electrophoretic migration at pH 8.6 in the 2 ml. microanalytical cell for 14,400 seconds under a potential gradient of 6.45 volt/cm.

The ascending and descending boundaries of the supernate from the pH 4.5 equilibrated RS-1 solids were brought to the midpoint of the center section of the 11 ml. analytical cell and electrophoretic migration conducted for 14,400 seconds under a potential gradient of 6.6 volt/cm. Photographs were made at hourly intervals. The protein solution was then recovered from the cell, and in some instances then equilibrated with veronal buffer by dialysis, and subjected to a repeat electrophore-

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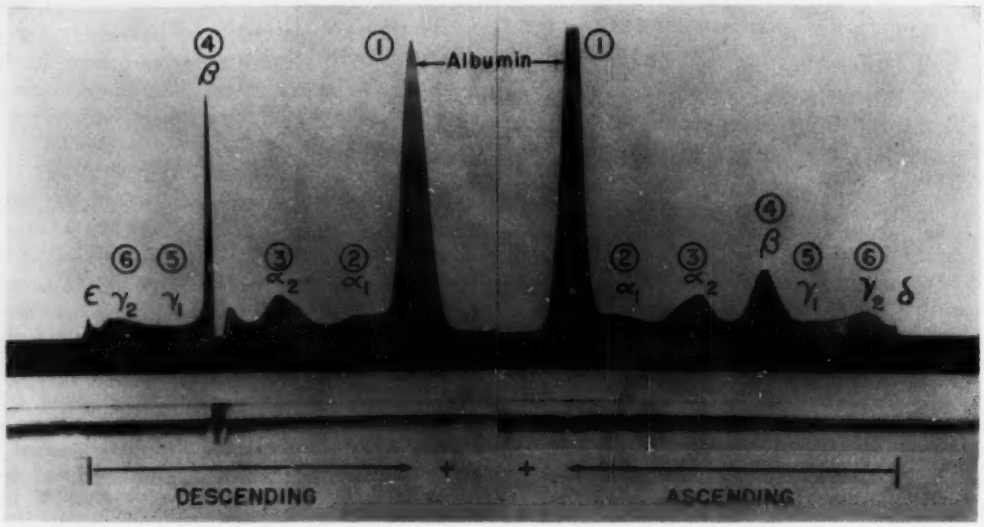

FIG. 1. ELECTROPHORETIC CONCENTRATION GRADIENTS OF NORMAL HUMAN SERUM

Separation in veronal buffer, ionic strength 0.1, pH 8.6, at 1° C., under a potential gradient of 5.6 volt/cm. for 14,400 seconds. Concentration gradients are numbered in order of decreasing mobility toward the anode.

sis under the conditions described for veronal-buffered proteins above.

Three pools of RS-1 from urine collections from 12 subjects were recovered in the lyophilized state. The dry weights were determined and a 0.1 aliquot (approximately 180 mg.) was removed for electrophoresis at pH 8.6 and 4.5 as described for the individual RS-1 samples above. Each was then separated into fractions RS-1A

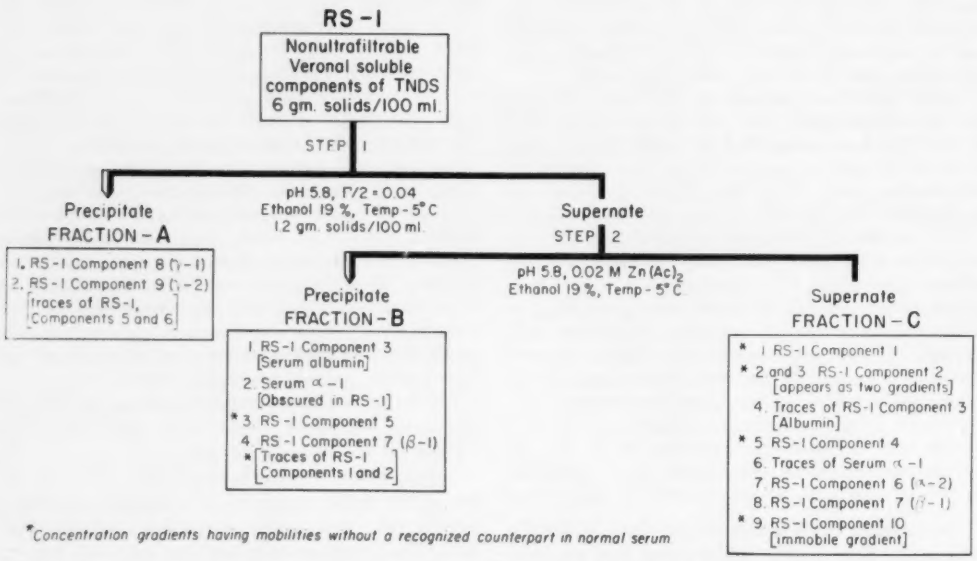
RS-I
Nonultrafiltrable Veronal soluble components of TNDS 6 gm. solids/100 ml.
STEP 1
pH 5.8, Γ/2 = 0.04 Ethanol 19 %, Temp - 5° C 12 gm. solids/100 ml.
Precipitate FRACTION - A
1. RS-I Component 8 (γ-1)
2. RS-I Component 9 (γ-2)
[traces of RS-I, Components 5 and 6]
Supernate
STEP 2
pH 5.8, 0.02 M Zn(Ac)2 Ethanol 19 %, Temp - 5° C
Precipitate FRACTION - B
1. RS-I Component 3 [Serum albumin]
2. Serum α-1 [Obscured in RS-I]
* 3. RS-I Component 5
4. RS-I Component 7 (β-1)
* [Traces of RS-I Components 1 and 2]
Supernate FRACTION - C
* 1. RS-I Component 1
* 2 and 3. RS-I Component 2 [appears as two gradients]
4. Traces of RS-I Component 3 [Albumin]
* 5. RS-I Component 4
6. Traces of Serum α-1
7. RS-I Component 6 (α-2)
8. RS-I Component 7 (β-1)
* 9. RS-I Component 10 [immobile gradient]
*Concentration gradients having mobilities without a recognized counterpart in normal serum

FIG. 2. CONCENTRATION GRADIENTS IDENTIFIED ELECTROPHORETICALLY AT pH 8.6 IN THE THREE SUBFRACTIONS OF RS-1 SOLIDS

(Cohn I, II and III), RS-1B (Cohn IV and V), and RS-1C (Cohn VI) by a modification of Cohn's Method 10, as previously described.

The wet precipitates of RS-1A were taken up in 3.5 ml. of veronal and equilibrated against the buffer by dialysis. Electrophoresis was then carried out in the 2 ml. microanalytical cell for 14,400 seconds under a potential gradient of 5.6 volt/seconds. This material was not studied electrophoretically at pH 4.5, since practically all of the protein precipitated spontaneously during the equili-

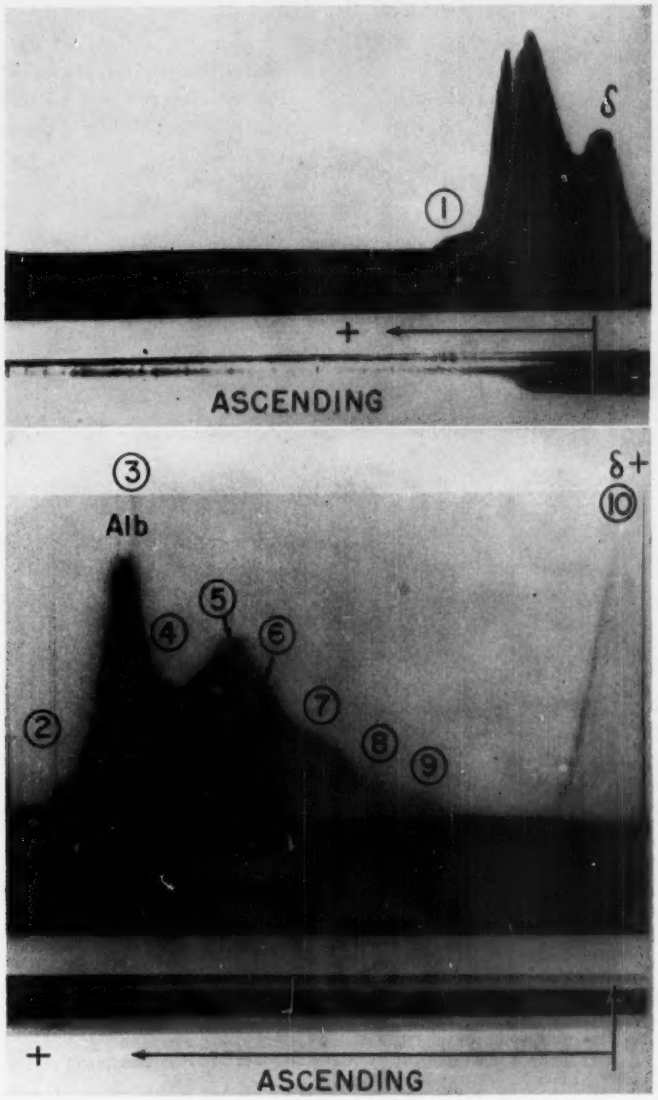

FIG. 3. CONCENTRATION GRADIENTS OF POOLED TOTAL RS-1 SOLIDS (POOL 14) IDENTIFIED ELECTROPHORETICALLY

Separation in veronal buffer, ionic strength 0.1, pH 8.6, at 1° C., under a potential gradient of 5.6 volt/cm. (A) After migration for 3,600 seconds to demonstrate gradient 1; (B) after migration for 14,400 seconds (gradient 1 is beyond the optical range).

bration dialysis with acetate buffer. This behavior was not altered by taking up the RS-1A precipitates in 15 ml. of veronal and concentrating to a volume of 3.5 ml. by lyophilization. A similar fraction of plasma proteins (Cohn I, II and III) obtained by the same technique as RS-1A showed no significant precipitation on dialysis to pH 4.5, but electrophoresis at pH 8.6 indicated many more components than the urinary RS-1A. Commercial human gamma globulin was also soluble in the pH 4.5 buffer at concentrations of 1 to 2 per cent.

The wet precipitates of RS-1B (Cohn IV and V) were taken up in veronal buffer sufficient to give a protein concentration of 1.3 to 1.5 per cent weight/volume. Electrophoresis at pH 8.6 and 4.5 was done as described above. A precipitate obtained on centrifugation of the pH 4.5 equilibrated RS-1B was subjected to electrophoresis at pH 8.6 as described for the whole RS-1 fraction.

The RS-1C (Cohn VI) components were recovered from the 260 ml. of filtrate by dialysis against distilled water and subsequent lyophilization. The dry solids were then suspended in veronal buffer to give a concentration of 1.5 per cent (weight/volume) and electrophoresis at pH 8.6 and 4.5 was accomplished as above. No precipitate was obtained on centrifugation of the pH 4.5 equilibrated RS-1C.

RESULTS

Veronal buffer, pH 8.6.

The concentration gradients consistently identified in each fraction were numbered in order of decreasing mobility toward the anode. The method of numbering is illustrated with the familiar pattern of normal serum in Figure 1. A flow sheet of those gradients appearing in the various Cohn fractions at pH 8.6 is presented in Figure 2. In veronal buffer at pH 8.6, a total of 10 concentration gradients was consistently identified in RS-1 (Figure 3). The relative concentration (area) of each gradient was determined by planimetry (Table I). The relative concentrations of the various gradients were quite constant for the three pooled samples; seven of the 11 individual RS-1 samples were within the range of variation encountered in the pooled specimens. Four of the individual RS-1 samples were found to have variations in area in gradients 5, 6 and 10 which were greater than one standard deviation from these gradients of the pooled samples. Representative examples of the archetypal and variant electrophoretic patterns of individual samples of RS-1 are presented in Figure 4.

The mobility relations of these RS-1 concentration gradients to those of normal serum under identical conditions of electrophoresis and mode of calculation are illustrated in Table II and Figures 5, 6 and 7. Note that the mobilities of serum components are approximately 10 per cent greater than those reported by Dole (4). This is due to the necessity of measuring distances of descending migration from the trailing margin of the initial protein buffer boundary, and possibly to slight mechanical variations in the optical systems and instruments.

RS-1 concentration gradient 1 was recognized as the "x"-component by Rigas and Heller (5) (Figure 3A). Concentration gradients 1 and 2 were previously designated as fast components in this laboratory (6). These components appear in both RS-1B and RS-1C fractions but are more clearly delineated in RS-1C, in which they may be relatively more concentrated. The appearance of RS-1 gradient 2 as two gradients (RS-1C gradients 2 and 3) in fraction C is not unexpected, since this gradient tended to spread in RS-1 and was relatively more concentrated in RS-1C.

Paper electrophoresis at pH 8.6, with commercial chondroitin sulfate as a control and aqueous toluidine blue O for development, has suggested the presence of two major components showing γ-metachromasia in RS-1, and in both RS-1B and RS-1C fractions. The faster component had a mobility slightly less than commercial chondroitin sulfate and the second component was

TABLE I

Average percentage of RS–1 present in each concentration gradient on separation at pH 8.6, as determined planimetrically from both ascending and descending patterns*

Gradient No.	2	3	4	5	6	7	8	9	10
Mean	2.64	25.93	4.63	32.30	12.68	10.10	6.02	5.69	17.46†
Range	1.3–6.6	14.5–35.9	2.6–8.4	22.6–43.2	7.8–23.0	6.2–16.3	2.9–9.8	3.3–9.6	2.8–47.6
S. D.	1.20	6.28	1.46	6.48	3.68	2.70	1.83	2.05	13.23

* Data from patterns for 11 individual and three pooled specimens.
† Per cent of total pattern. Figures for gradients 2 through 9 represent per cent of total, *exclusive* of gradient 10.

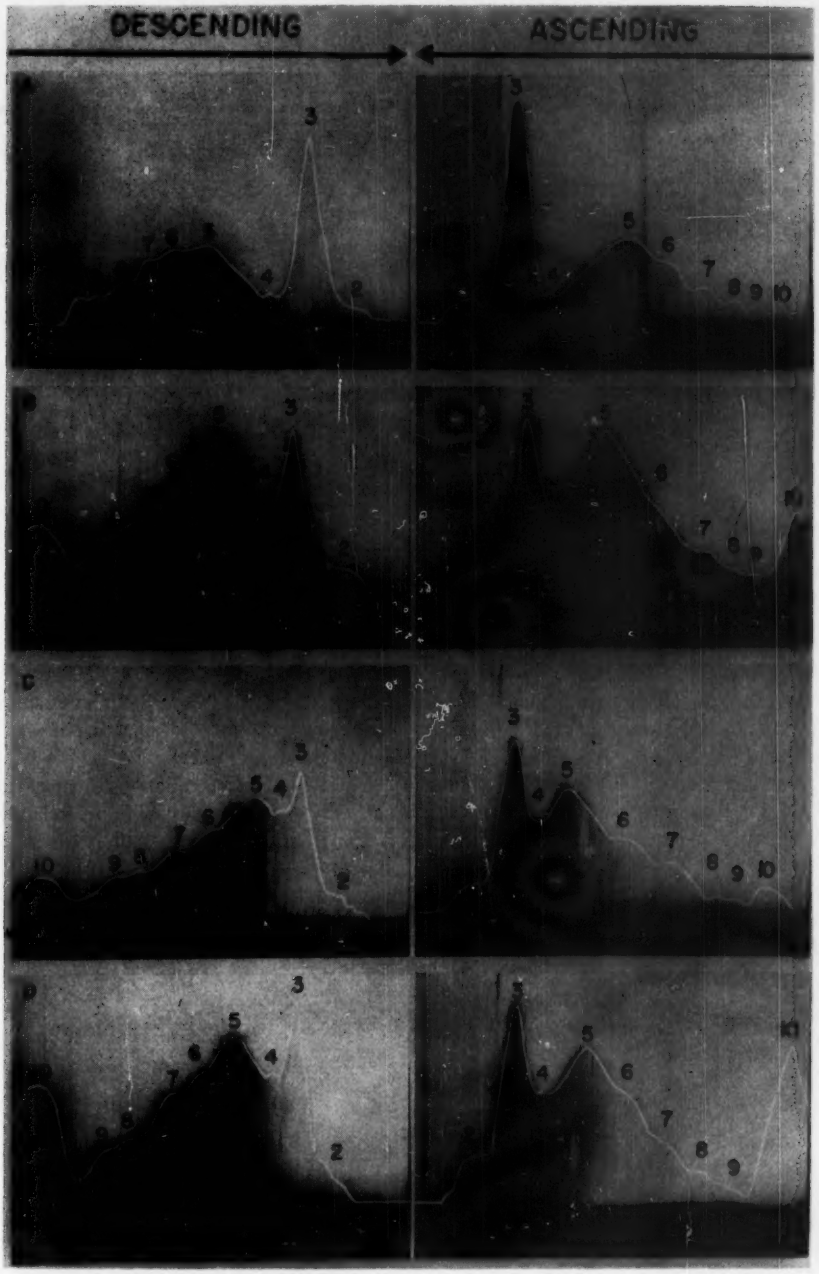

FIG. 4. CONCENTRATION GRADIENTS OF TOTAL RS-1 SOLIDS FROM 72 HOUR URINE SPECIMENS FROM EACH OF FOUR SUBJECTS

Conditions of electrophoresis as in Figure 3B. (A) and (B) Extreme variations encountered in relative areas of various gradients; (C) and (D) archetypal distribution of various concentration gradients.

TABLE II

Concentration gradients appearing in serum and in RS-1 fraction of urine TNDS at pH 8.6 *

		Descending toward Anode; Negative Mobility $-\mu \times 10^{-5}$ cm.^{2}/volt sec.										
Normal Serum	Component			① Albumin		② α-1		③ α-2	④ β	⑤ γ-1	⑥ γ-2	⑦ ε Buffer-protein gradient
	μ mean ± s.d.			6.64 ±0.14		5.45 ±0.17		4.34 ±0.24	3.22 ±0.22	2.42 ±0.24	1.55 ±0.23	
Normal Urine RS-1	Component	①	②	③	④		⑤	⑥	⑦	⑧	⑨	⑩ Immobile Component
	μ mean ± s.d.	10.24 ±0.14	7.79 ±0.13	6.76 ±0.12	6.06 ±0.17		4.88 ±0.36	4.02 ±0.43	3.31 ±0.32	2.41 ±0.24	1.72 ±0.24	
COHN FRACTIONS												
RS-1A	Component						Trace	Trace		①	②	
	μ mean ± s.d.									2.38 ±0.20	1.61 ±0.21	
RS-1B	Component	Trace	Trace	①		②	③		④			
	μ mean ± s.d.			6.76 ±0.11		5.64 ±0.26	4.85 ±0.38		3.10 ±0.21			
RS-1C	Component	①	②	③	④	⑤	⑥	⑦	⑧			⑨ Immobile Component
	μ mean ± s.d.	10.40 ±0.16	8.68 ±0.20	7.39 ±0.14	6.83 [Trace]	6.25 ±0.20	5.68 ±0.28	4.52 ±0.46	3.24 ±0.29			

* Moving boundary electrophoresis: veronal buffer, pH 8.6, 0.1 M; electropotential 5.6 volt/cm.; time 14,400 seconds. The sum of planimeter units (area) of blocked components accounts for more than 95 per cent of the recovered portion of each fraction. Gradients of each fraction are numbered in order of decreasing mobility toward anode.

only a little slower, although considerable trailing makes such interpretation rather ambiguous. Whether these represent the gradients 1 and 2 of RS-1 is unknown. However, traces of these components appeared in both RS-1B and RS-1C with the greater concentration of both appearing in RS-1B. The mobilities on paper relative to albumin were consistent with RS-1 gradients 1 and 2.

RS-1 concentration gradient 3 had the same mobility as serum albumin at pH 8.6 and 4.5. It also responded to the Cohn Method of fractionation by appearing predominantly in RS-1B (Cohn Fractions IV and V).

RS-1 concentration gradient 4 appeared in good concentration in fraction RS-1C as gradient 5. This gradient may be present in such small concentration in human plasma that it was obscured by the albumin gradient.

The RS-1 concentration gradients 5 and 6 were not only the most variable in relative concentration but also in electrophoretic mobility. In a previous report the mobility of component 5 was found to have a mean value of -4.81×10^{-5} cm.^{2} per volt second, as compared with the present determination of -4.88×10^{-5}, and component 6 was found to have a mean value of -3.92×10^{-5}, as compared with -4.02×10^{-5} for the

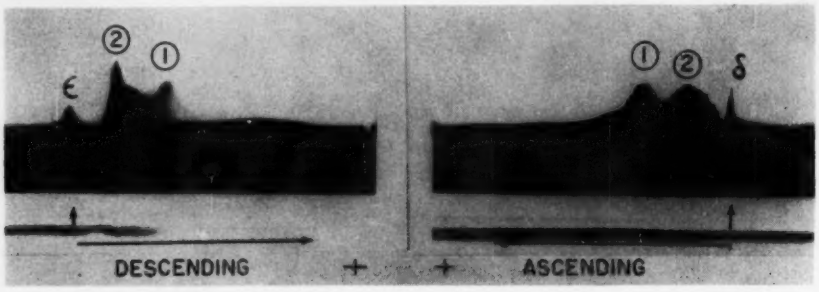

FIG. 5. CONCENTRATION GRADIENTS OF SUBFRACTION RS-1A (POOL 14, FIGURE 3)
Conditions of electrophoresis as in Figure 3B.

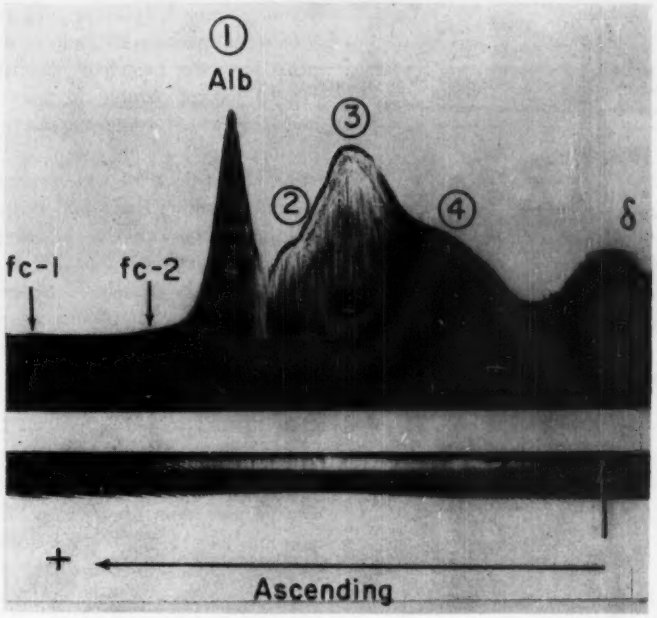

FIG. 6. CONCENTRATION GRADIENTS OF SUBFRACTION RS-1B (POOL 14, FIGURE 3)

Conditions of electrophoresis as in Figure 3B. Gradients fc-1 and fc-2 are too small to be measured planimetrically.

present study (6). Component 5 thus has a mobility intermediate between serum alpha-1 and alpha-2. A gradient of mobility similar to RS-1 gradient 5 appeared in RS-1B as component 3. A gradient of mobility consistent with serum alpha-1 appeared in both RS-1B (gradient 2) and in RS-1C (gradient 6). These gradients of alpha-1 mobility may have been obscured in RS-1 by the large gradient 5, or their mobility may have been altered by other components of the various fractions so that they appeared as gradient 5 in RS-1.

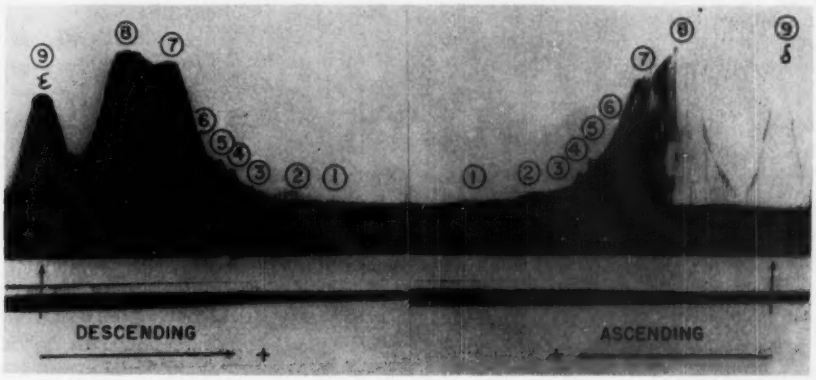

FIG. 7. CONCENTRATION GRADIENTS OF SUBFRACTION RS-1C (POOL 14, FIGURE 3)

Conditions of electrophoresis as in Figure 3B.

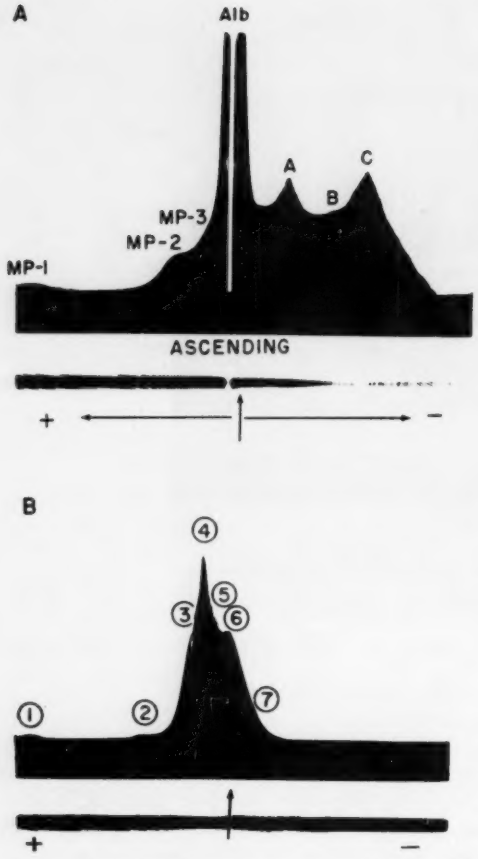

FIG. 8. ELECTROPHORETIC CONCENTRATION GRADIENTS FOLLOWING SEPARATION IN ACETIC ACID-ACETATE BUFFER

Ionic strength 0.1, pH 4.5, at 1° C., under a potential gradient of 6.6 volt/cm., after migration for 14,400 seconds. (A) Normal human serum; (B) pooled total RS-1 solids (Pool 14, Figure 3).

RS-1 gradient 6 had a mobility consistent with serum alpha-2 globulins and appears in the RS-1C (Cohn VI) fraction as gradient 7. Cohn Fraction VI contains serum alpha-2 protein.

RS-1 gradient 7 had a mobility consistent with that of serum beta-1 and appeared as gradient 4 in RS-1B and as gradient 8 in RS-1C. Fraction RS-1B corresponded to Cohn Fractions IV and V, which contain the serum beta-1 metal-combining protein; fraction RS-1C corresponded to Cohn Fraction VI, which contains serum beta-1 protein.

RS-1 gradient 8 corresponded in mobility to serum gamma-1 (plasma phi) and RS-1 gradient 9 to serum gamma-2. Both of these gradients appeared as the principal constituents of RS-1A. RS-1A is comparable to Cohn Fractions I, II and III, which contain gamma globulin and fibrinogen.

RS-1 gradient 10 was a very large stationary boundary, which Rigas and Heller (5) recognized as the "polysaccharide plus epsilon gradient." This large gradient appeared in the RS-1C fraction of urinary proteins. No such substance has been recognized in plasma or in Cohn Fraction VI, which is comparable with RS-1C.

Acetate buffer, pH 4.5

Mehl, Humphrey and Winzler (7) have demonstrated that normal human serum contains three components which retain a net negative charge and have negative mobilities greater than albumin at pH 4.5. These substances have been labeled as mucoproteins MP-1 (orosomucoid), MP-2 and MP-3. Albumin retains a small net negative charge and migrates toward the anode. The remainder of the serum proteins have a net positive charge and appear as three concentration gradients, A, B and C, designated in order of increasing mobility toward the cathode (Figure 8A).

Table III presents a comparison of the mobilities of various concentration gradients in normal serum and in the RS-1 urinary proteins. The mobilities of the serum mucoproteins have been reported to be slightly different when determined on isolated mucoprotein (7) than on mucoprotein in the presence of serum proteins (8).

The total RS-1 fraction was found to contain six distinct gradients with net negative charges at pH 4.5 (Figure 8B). Only a small gradient with mobility comparable to serum component A (RS-1 gradient 7) was found to have a mobility toward the cathode. By planimetry this gradient accounted for approximately 5 per cent of the total RS-1. This component of net positive charge appeared unchanged in fraction RS-1B, as gradient 4 (Figure 9).

Gradient 1 of RS-1 had a mobility much greater than any yet identified in whole serum and appears unchanged as gradient 2 in fraction RS-1C (Figure 10). Gradients 1 and 3 of RS-1C were not identified in the total RS-1, but were consistently present in RS-1C.

TABLE III

*Concentration gradients appearing in serum and in RS-1 fraction of urine TNDS at pH 4.5**

		−Mobility, toward Anode							+Mobility, toward Cathode		
Normal Serum	Component			① MP-1		② MP-2	③ MP-3	④ Albumin	⑤ glob. A	⑥ glob. B	⑦ glob. C
	μ mean ± s.d.			−3.98 ± 0.24		−1.25 ± 0.14	−0.78 ± 0.08	−0.29 ± 0.03	+0.84 ± 0.08	+1.59 ± 0.10	+2.26 ± 0.09
RS-1	Component	①		②	③	④	⑤	⑥	⑦		
	μ mean ± s.d.	−10.40 ± 0.18		−4.78 ± 0.29	−2.09 ± 0.21	−1.27 ± 0.17	−0.82 ± 0.10	−0.28 ± 0.05	+0.85 ± 0.07		
COHN FRACTIONS											
RS-1B	Component				①	②	?	③	④		
	μ mean ± s.d.				−2.16 ± 0.32	−1.20 ± 0.25	Obscured	−0.30 ± 0.04	+0.88 ± 0.05		
RS-1C	Component	①	②	③	④	⑤					
	μ mean ± s.d.	−15.35 ± 0.27	−10.80 ± 0.17	−7.30 ± 0.13	−4.80 ± 0.32	−1.36 ± 0.10					

* Moving boundary electrophoresis: acetate buffer, pH 4.5, 0.1 M; electropotential 6.6 volt/cm.; time 14,400 seconds. Mobility expressed as − or + $\mu \times 10^{-5}$ cm.^{2} per volt second. Gradients of each fraction are numbered in order of decreasing mobility toward anode. S.D. = standard deviation of determination from 11 subjects for RS-1, three or more for each fraction.

Gradient 2 of RS-1 was equivalent in mobility to serum MP-1. Mehl, Golden and Winzler reported a mobility of -4.2×10^{-5} for this component in combination with serum proteins (8), which is in good agreement with the value he obtained for isolated mucoprotein MP-1. This material appeared unchanged as gradient 4 in fraction RS-1C.

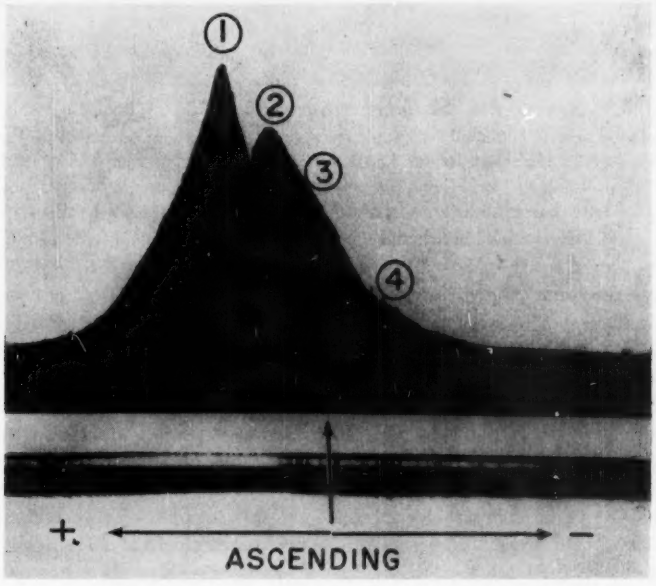

FIG. 9. CONCENTRATION GRADIENTS OF SUBFRACTION RS-1B (POOL 14, FIGURE 3)

Conditions of electrophoresis as in Figure 8B.

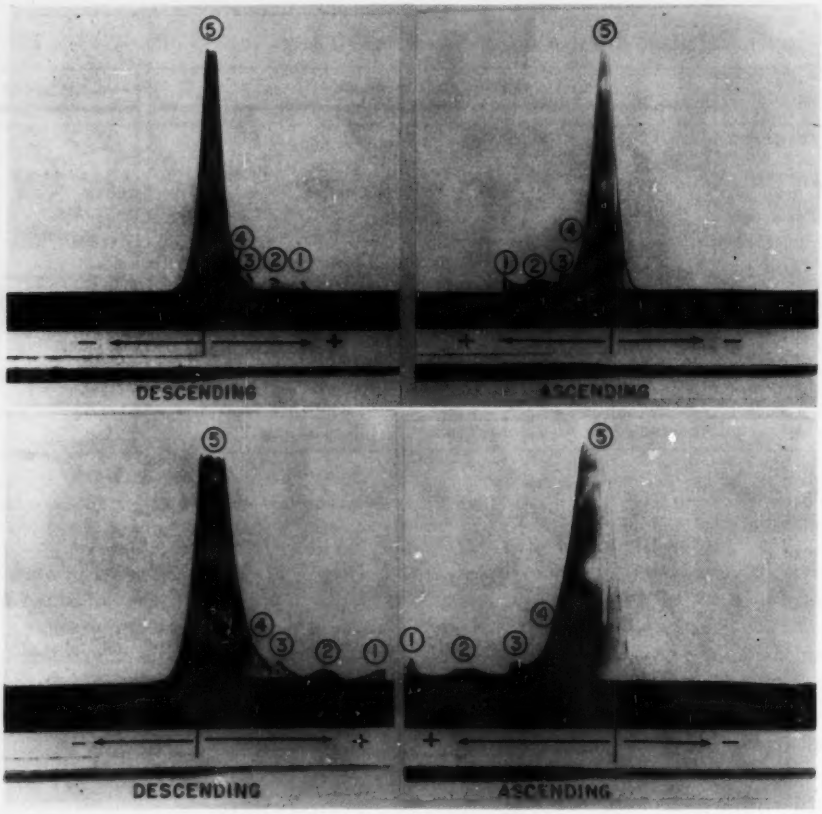

FIG. 10. CONCENTRATION GRADIENTS OF SUBFRACTION RS-1C (POOL 14, FIGURE 3)
Separation in acetate buffer, pH 4.5, ionic strength 0.1, at 1° C., under a potential gradient of 2.6 volt/cm. (A) After migration for 5,520 seconds; (B) after migration for 9,960 seconds.

Gradient 3 of RS-1 had a mobility consistent with isolated serum MP-2, which Mehl and coworkers reported as -2.4 to -2.8×10^{-5} (7). This gradient appeared unchanged in fraction RS-1B as gradient 1.

Gradient 4 of RS-1 had a mobility more consistent with serum MP-2 as determined in the presence of serum proteins (8). Gradients of corresponding mobility appeared in the RS-1B as gradient 2 and in RS-1C as gradient 5. By planimetry, gradient 5 accounts for 74 per cent of fraction RS-1C, a value comparable to the combined area of gradients 5, 7, 8 and 9 of this fraction at pH 8.6. The gradient 5 of RS-1C at pH 4.5 is thus of much greater concentration than the total MP-2 of RS-1 even when RS-1 gradients 3 and 4 are combined.

Gradient 5 of RS-1 was comparable to serum MP-3 in mobility; Mehl, Humphrey and Winzler find a value of -0.5 to -1.2×10^{-5} for isolated MP-3 (7). This gradient is absent from the RS-1C fraction and is probably obscured by albumin and gradient 2 in the RS-1B fractions.

RS-1 component 6 is considered to correspond to serum albumin and appeared in the RS-1B fraction at pH 4.5 and 8.6.

The precipitates which appeared in the total RS-1 and the RS-1B fractions on dialysis from pH 8.6 to pH 4.5, were found to account for approximately 15 to 20 per cent of the total solids. Electrophoresis of these precipitates at pH 8.6 revealed the presence of all concentration gradients in approximately the same relative concentration as in the original preparations. It was noted that

the precipitates from RS-1B contained a slightly lower albumin to globulin ratio than did the pH 4.5 soluble supernates. Albumin accounted for 15.5 to 16.1 per cent of the area of the supernate RS-1B and 12.8 to 14.1 per cent of the precipitate of RS-1B. This difference was due largely to a slight increase of gradients 2 and 3 (Figure 6) in the precipitates as compared with the supernates. The separation of solids during equilibrium dialysis to pH 4.5 is therefore considered to represent a coprecipitation involving all components of the solids in essentially the same ratio as their concentration in the solution. This remarkable behavior of such diverse proteins with respect to solubility change with variation in pH was also observed by Mehl and Golden (9). Although the precipitate which they obtained from a similar treatment of serum was proportionately less than that which we obtained with urine proteins, they were satisfied that no major portion of one component was being removed in the precipitate.

DISCUSSION

Previously reported studies of fraction R-1 of urinary TNDS (10) and studies of fraction UF-O now being prepared for publication have convinced us that neither of these fractions contains detectable quantities of the protein components recognizable by conventional boundary electrophoresis of plasma. Any such serum protein components which escape, or are secreted, into the urine must therefore appear in the RS-1 fraction of TNDS. This precludes neither the possibility that all or part of the UF-O (total ultrafiltrable) fraction is derived from the blood plasma nor the possibility that some components of RS-1 originate from sources other than plasma (*e.g.*, genital, urinary or nephrogenic epithelial secretions).

The normal human serum contains approximately 121 mg. per 100 ml. of protein bound hexose, 83 mg. per 100 ml. of hexosamine, 8.9 mg. per 100 ml. of methylpentose and 60 to 66 mg. per 100 ml. of sialic acid (11). The majority of these are constituents of mucosubstances and both the analytical (2) and the above electrophoretic data indicate that the bulk of the RS-1 fraction of urine is composed of such mucosubstances. All of the principal fractions of plasma proteins except V obtained by the Cohn fractionation technique have been demonstrated to contain mucosubstances (12, 13). Of those concentration gradients commonly recognized by electrophoresis of blood plasma at pH 8.6, all have been demonstrated to contain mucosubstances (14). Albumin has been obtained in a carbohydrate-free form but the difficulty in achieving this suggests that mucosubstances may be closely associated with albumin in the electrophoretic spectrum. Many of the mucosubstances have been isolated in relatively pure form and the following have received considerable attention in recent years:

Orosomucoid (alpha-1 mucoprotein, MP-1) has an isolectric point of approximately 2.7 (11, 15, 16). Alpha-2 mucoproteins include at least three electrophoretically homogenous components, all with isoelectric points of 3.7 to 4.4 (7, 17). Haptoglobulin has an electrophoretic mobility similar to the alpha-2 globulins and has been recovered from human serum and nephrotic urine (14). Both pituitary and chorionic gonadotropins have mobilities in the range of the alpha globulins and isoelectric points of near 3.2 (18). Thyroxin binding protein of human serum is electrophoretically a component of the alpha-2 globulins at pH 8.6 and retains a net negative charge at pH 4.5 (19). Prothrombin contains bound hexose, hexosamine and sialic acid (14) and migrates with the alpha globulins at pH 8.6. Plasma cholinesterase is considered to be a glycoprotein with electrophoretic mobility similar to the alpha-2 globulins (20). Beta-1-metal-combining protein (siderophilin) was crystallized (21) and has an isoelectric point near 5.8. Both the gamma-1 (19 S) and gamma (7 S) globulins were found to contain hexose, fucose, hexosamine and sialic acid (22).

There are thus ample mucosubstances in normal plasma to account for the "globulin" components of RS-1 urinary proteins at pH 8.6. Present knowledge does not permit an unequivocal identification of the various mucosubstances of RS-1 with those of plasma. It has been suggested that the reversal of the albumin/globulin ratio of urinary proteins is due to passage of small quantities of serum proteins through the glomeruli with preferential tubular reabsorption of albumin (23). The present studies indicate that all serum proteins which pass the normal nephron have very

low isoelectric points, all but 5 per cent retaining a net negative charge at pH 4.5. The observation that the relative concentration of mucosubstances in the electrophoretic gradients of serum are proportionately the same as in gradients of corresponding mobility in urinary RS-1 suggests that these molecules pass the glomerular membrane in ratios approximating their plasma concentration. Whether tubular reabsorption plays any significant part in the final urinary concentration or composition remains unknown. The absence of the major plasma globulins with isoelectric points more than 4.5 suggests that under normal conditions RS-1 components appear in the urine as a result of selective glomerular filtration, passage through the membrane being related not alone to molecular size and shape but also in some manner to electrical charge or configuration.

SUMMARY

The RS-1 (nonultrafiltrable and veronal buffer soluble) fraction of urinary proteins has been subjected to electrophoretic analysis at pH 8.6 and 4.5. This fraction contains all of the serum albumin present in urine and thus presumably all serum proteins of molecular size greater than albumin. The minimal molecular weight component in this fraction has not been identified, but the majority of the components are of such size that diffusion is not a problem in conventional boundary electrophoresis of five hours' duration.

Cohn method 10 for fractionation of serum proteins at low temperature in the presence of low concentrations of ethanol and of zinc acetate has been applied to the RS-1 fraction. Three reproducible subfractions were obtained: RS-1A (Cohn I, II and III), RS-1B (Cohn IV and V), and RS-1C (Cohn VI). These fractions have also been analyzed by electrophoresis at pH 8.6 and 4.5.

Essentially all protein components which appear in normal urinary RS-1 solids have isoelectric points below pH 4.5. It is apparent that the majority, if not all, of the RS-1 solids are mucosubstances which have counterparts in the blood plasma.

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ELECTROLYTE AND ACID–BASE BALANCE DURING ACUTE LOADING WITH RUBIDIUM CHLORIDE * †

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Previous studies have shown that administration of rubidium to normal or potassium-depleted rats results in *a*) metabolic acidosis (1), and *b*) accumulation of rubidium in muscle tissue where it attains final concentrations considerably higher than that of potassium (2). Since it was found that rubidium was at least as effective as potassium in lowering the plasma CO_2 content of potassium-deficient nephrectomized animals (1), it was suggested that administered rubidium, like potassium, probably exchanges with intracellular hydrogen.[1]

In view of these facts it was deemed of interest to obtain information about the simultaneous renal and tissue exchanges of electrolytes during loading with rubidium. Short term balance studies were therefore carried out on rats given 40 mEq. per Kg. of RbCl over two days, and the results compared with those in control animals given an equivalent amount of KCl.

The data to be reported show that acute loading resulted in the rapid accumulation of very large amounts of rubidium in tissues with the simultaneous displacement of an almost equal amount of intracellular cation. Approximately half of the estimated cellular content of potassium was excreted in the urine together with a smaller quantity of sodium. A severe metabolic acidosis developed which was unaccompanied by compensatory increase in the urinary excretion of acid, thus demonstrating a renal defect which perpetuates the acidosis.

METHODS

Male albino rats of the Sprague-Dawley strain, weighing approximately 300 Gm., were used in all experiments. Four day balance studies were carried out, a two day control period on a standard liquid diet being followed by a two day period during which RbCl or KCl was added to the food. The composition of the diet fed during the control period is shown in Table I, its caloric value being approximately 2.5 calories per ml. The diet was administered by means of a stomach tube in four equal feedings per day; 87 ml. of diet per Kg. of weight was given, this being the largest quantity which could be administered without producing severe diarrhea. After 48 hours on the control diet the rats were divided into two groups. Group I, which consisted of 10 rats, was given a supplement of KCl, 20 mEq. per Kg. per day for the next two days, in addition to the standard control diet. Group II, consisting of 11 rats, received the control diet plus 20 mEq. per Kg. per day of RbCl to which tracer quantities of Rb^{86} had been added. Thus the total load of rubidium or potassium for the two days of loading was 40 mEq. per Kg., an amount approximating the total quantity of potassium in the body. Tap water *ad libitum* was provided for drinking purposes throughout the experiment.

To facilitate complete separation of urine and feces the animals were immobilized in light plaster casts. A modification of the cast described by Lathrop and Harper (4) was used, the chamber for the collection of expired gases being omitted from the design. The animals in

TABLE I

Composition of liquid diet

Glucose	344 Gm./L.
Mazola Oil®	105 Gm./L.
Casein	50 Gm./L.
Sodium	27 mEq./L.
Potassium	10 mEq./L.
Chloride	28 mEq./L.

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[1] As recently pointed out by Eckel, Norris and Pope (3), the administered potassium may restore extracellular CO_2 content to normal by the displacement of intracellular cationic amino acids which accumulate in potassium-depleted tissues.

TABLE II

*Plasma composition**

Group	Na	K	Rb	Rb + K	CO_2	Cl
	mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mMoles/L.*	*mEq./L.*
I (K)	144 ± 2.11	3.9 ± 0.58			23.9 ± 1.33	104 ± 3.46
II (Rb)	153 ± 6.41	2.6 ± 0.84	1.3 ± 0.24	3.9 ± 1.07	13.2 ± 4.83	114 ± 3.90

* Mean values ± S. D.

their casts were strapped onto a frame designed to permit the hind quarters of the rat to project over the edge. Urine was collected under mineral oil in a glass cylinder held in position beneath the penis, phenyl mercuric nitrite and toluene being used as a preservative. Stools were collected in a beaker suspended under the anus.

On the morning of the fifth day—about 16 hours after the last feeding—the animals were anesthetized with the intraperitoneal injection of a 2 per cent solution of Amytal Sodium® (approximately 0.75 ml. per 100 Gm. weight) and then exsanguinated by withdrawal of blood from the abdominal aorta into a heparinized syringe.

Total CO_2 content of the plasma and urine was estimated by standard manometric technique, and chloride in stool and diet was determined by a modified Volhard titration. Urine pH was determined at room temperature with a glass electrode, urine ammonia by the permutit method of Folin, and total titratable acid by titration to pH 7.4 with standard alkali. Sodium analyses were carried out with a flame photometer, as were analyses for potassium in those specimens in which no rubidium was present. For samples containing mixtures of rubidium and potassium a special analytical technique was devised based on the combined use of tracer Rb^{86} and the chloroplatinate titration. This is described elsewhere (2).

RESULTS

During the course of the experiment the potassium-loaded animals (Group I) lost, on the average, 43.6 ± 6.7 Gm. weight; the rubidium-loaded rats (Group II) had a mean weight loss of 50.8 ± 8.2 Gm., a value which is not significantly different.[2] The only obvious evidence of toxicity was the development of signs of increased irritability in most of the rubidium-treated animals.

The data on plasma composition at the end of the experiment are summarized in Table II. It will be seen that the rubidium animals had a striking metabolic acidosis, the mean value for the plasma CO_2 content in this group being 13.2 ± 4.83 mMoles per L. This was significantly lower than the mean CO_2 content of the rats loaded with potassium (23.9 ± 1.33 mMoles per L.). Plasma potassium in the potassium-loaded animals was 3.9 ± 0.58 mEq. per L. In the rubidium-loaded group the potassium concentration was 2.6 ± 0.84 mEq. per L., while the rubidium concentration was 1.3 ± 0.24 mEq. per L. Mean sodium and chloride concentrations in the rubidium-loaded group were slightly above normal and significantly higher than in the potassium group.

The external balance data for sodium, potas-

[2] Differences described as "significant" had a "p" value less than 0.01, unless stated otherwise.

TABLE III

*External balance data** †

Group	Na		K		Rb	Cl	
	Cumulative balance during control (days 1 and 2)	Cumulative balance during loading (days 3 and 4)	Cumulative balance during control (days 1 and 2)	Cumulative balance during loading (days 3 and 4)	Cumulative balance during loading (days 3 and 4)	Cumulative balance during control (days 1 and 2)	Cumulative balance during loading (days 3 and 4)
	mEq./Kg.		*mEq./Kg.*		*mEq./Kg.*	*mEq./Kg.*	
I (K)	+0.6 ± 1.21	−1.7 ± 0.37	−5.4 ± 0.77	− 0.7 ± 6.42		+1.8 ± 1.11	+5.6 ± 5.41
II (Rb)	+0.9 ± 3.19	−4.8 ± 0.93	−5.8 ± 1.72	−27.9 ± 4.75	+29.3 ± 2.29	+2.1 ± 1.88	+4.0 ± 5.39

* Do not include stool data (see text).
† Mean values ± S. D.

sium, rubidium and chloride are set out in Table III expressed, for purposes of comparison, as mEq. per Kg. of initial body weight.

It should be noted here that stool electrolytes were not included in the calculation of the balance data. However, complete electrolyte analyses were carried out on stools obtained from four randomly selected animals in Group I and from six in Group II, during control and loading periods. Electrolyte content was found to be negligible and stool analyses were therefore omitted in the other animals. For each electrolyte, where appropriate, two sets of mean data are presented. In the first column are shown the figures for the cumulative balance at the end of the second control day immediately prior to loading. The second column shows the cumulative balance for the two days of loading (Days 3 and 4). During the two day control period no significant differences between the groups were observed. Sodium and chloride balances were not significantly different from zero in either group, and in both groups there was a small negative balance of potassium (Group I: 5.4 ± 0.77 mEq. per Kg.; Group II: 5.8 ± 1.72 mEq. per Kg.).

Over the two day loading period the animals given KCl were virtually in potassium balance, the cumulative value being -0.7 ± 6.42 mEq. per Kg. However, if it is assumed that the average rate of potassium loss during the first two days (probably associated with the weight loss and tissue breakdown caused by immobilization in a cast) would have continued during Days 3 and 4 in the absence of loading, then the observed figure for the loading period may represent a slight retention of potassium, approximately 10 per cent of the load. There was a small negative balance of sodium during the period of potassium-loading (-1.7 ± 0.37 mEq. per Kg.), but a positive balance of chloride ($+5.6 \pm 5.41$ mEq. per Kg.).

The rubidium-loaded rats in Group II retained an average of 29.3 ± 2.29 mEq. per Kg. of rubidium during Days 3 and 4, this amount being approximately 70 per cent of the administered load. This retention of rubidium was accompanied by a markedly negative potassium balance (-27.9 ± 4.75 mEq. per Kg.). Correction of this figure for the negative potassium balance during the control period would yield a net change in potassium of approximately -22 mEq. per Kg. during the period of rubidium loading.

There was a moderate negative balance of sodium during the two days of rubidium loading (-4.8 ± 0.93 mEq. per Kg.), this figure being significantly different from both the control balance during Days 1 and 2 and the balance during loading with KCl. There was no accompanying loss of chloride. Chloride balance during Days 3 and 4 was slightly positive ($+4.0 \pm 5.39$) but not significantly different from the balance during the control period or from the balance during loading with KCl.

Table IV summarizes the data on urinary excretion of ammonium, titratable acid and total CO_2. In the control periods there was no difference between the two groups with respect to ammonium, titratable acid or total CO_2 in the urine. Urine pH was virtually identical in both groups (range, 5.8 to 6.8). Net excretion of acid (roughly estimated as ammonium plus titratable acid minus CO_2 content) averaged about 6 to 7 mEq. per Kg per day.

During loading with KCl there were no significant changes in ammonium, titratable acid or CO_2

TABLE IV

*Urinary excretion of acid and base**

Group	Ammonium		Titratable acid		CO_2	
	Control period (days 1 and 2)	Loading period (days 3 and 4)				
	mEq./Kg./day		*mEq./Kg./day*		*mMoles/Kg./day*	
I (K)	4.6 ± 0.93	4.7 ± 1.28	1.7 ± 0.48	1.2 ± 0.50	0.4 ± 0.32	0.7 ± 0.41
II (Rb)	5.0 ± 1.32	3.5 ± 1.31	1.8 ± 0.63	1.3 ± 0.35	0.3 ± 0.33	0.2 ± 0.24

* Mean values ± S. D.

excretion and no appreciable change in urine pH. In the Group II animals which were loaded with RbCl there were no significant changes in the excretion of CO_2 or titratable acid when compared to the first two days or to the KCl loading period. On the other hand, ammonium excretion showed a small but significant reduction during the period of rubidium loading, when compared with the excretion during the control. The "p" value for the difference between ammonium excretion during loading with KCl and that during loading with RbCl was between 0.02 and 0.05. Urine pH did not change significantly during the loading period.

DISCUSSION

These experiments clearly demonstrate the remarkable avidity of the tissues of normal animals for administered rubidium and the speed with which a large proportion of body potassium can be replaced by this ion. Slightly more than 70 per cent of the rubidium load was retained (29.3 mEq. per Kg.). Since the final extracellular rubidium concentration was quite low (1.3 mEq. per L.), it can be reasonably assumed that most of the retained rubidium entered cells. On the basis of the simultaneous negative potassium balance of approximately half of total body potassium (22.1 mEq. per Kg., corrected for control), it would appear that the accumulated rubidium largely exchanged for intracellular potassium. Muscle tissue analyses in three randomly selected animals in Group II confirmed these exchanges in revealing approximately equal quantities of intracellular rubidium and potassium.

The finding of a low plasma concentration of potassium at the end of the period of rubidium loading (Table II) might suggest that the large kaluresis was the result of a primary increase in renal clearance of potassium with secondary tissue loss consequent to the hypokalemia. However, acute experiments in dogs given infusions of RbCl do not support this concept. These latter experiments revealed immediate rises in plasma potassium as rubidium entered cells (5). Increased renal excretion of potassium was shown to be the result of the hyperkalemia, there being no evidence for any direct effect of rubidium on renal tubular handling of potassium (6). Thus it would appear likely that in the present experiments transient elevations of plasma potassium were responsible for the kaluresis but had been dissipated in the 16 hours elapsing between the last load of rubidium and the time of blood sampling. The low plasma levels observed at this time presumably reflected the severe state of intracellular depletion which existed at the end of the study.

The large cellular uptake of rubidium was accompanied not only by potassium excretion but also by a diuresis of sodium which, although considerably smaller than that of potassium, was significantly larger than the sodium excretion of the Group I animals during the period of KCl loading. After correction for the sodium balance of the control period, there appeared to be a sodium loss of about 5.7 mEq. per Kg. during the rubidium-loading period. Unaccompanied as it was by any demonstrable loss of chloride, this diuresis of sodium probably represented additional intracellular cation displaced by the rubidium. As such, it could in part explain the observed discrepancy of approximately 7 mEq. per Kg. between rubidium uptake and corrected potassium loss. Additional support for this interpretation of the significance of the increased urinary sodium is derived from the finding of reduced intracellular sodium in the muscles of rubidium-loaded animals (2).

The powerful acidifying effect of rubidium was well demonstrated in the present experiments, the final plasma CO_2 content (13.2 ± 4.83 mMoles per L.) being significantly lower than that found in the potassium-loaded animals (23.9 ± 1.33 mMoles per L.). It has previously been shown in nephrectomized, potassium-deficient alkalotic rats that rubidium causes acidosis by the displacement of intracellular hydrogen (1).[3] Assuming constant endogenous acid production and a maximum value for extracellular volume of 300 ml. per Kg. body weight, transfer of only 3 mEq. per Kg. of hydrogen in the present experiments would have been required to produce the entire difference of 10 mEq. per L. in CO_2 content between the two groups. Together with the cell exchange of sodium discussed above, this relatively small displacement of hydrogen could approximately account for the entire discrepancy between the observed balance of rubidium and the corrected

[3] See footnote 1.

balance of potassium. However, the errors inherent in the balance measurements as well as in the correction of the potassium data are probably too large to permit accurate assessment of such small differences, and it is evident that these studies do not provide any definitive demonstration of cellular shifts of acid or their role in the pathogenesis of the acidosis.

On the other hand, these observations do permit certain conclusions about the role of the kidneys. It is clear from the unchanged urine CO_2 content in both groups during the loading period that there was no significant increase in bicarbonate excretion. Thus, renal loss of alkali played no role in the genesis of the acidosis in the rubidium-loaded animals. However, there appeared to be a slight reduction in excretion of acid due entirely to a small but consistent and significant reduction in ammonium excretion, as compared to the control period. If the endogenous production of acid were constant, this reduction in excretion of ammonium would represent the retention of a total of about 3 mEq. per Kg. of acid for the two day period of loading. To this extent, therefore, the kidneys might have contributed to the development of the acidosis.

Although the significance of the renal contribution to the pathogenesis of the extracellular acidosis in these experiments is somewhat uncertain, the data are unequivocal in demonstrating a failure of the kidneys to compensate for the acidosis. It is well known that rats are ordinarily capable of immediate and large increments in acid excretion (mainly ammonium) when challenged by an acidosis (7–9). It is apparent, therefore, that the kidneys of the Group II rats did not respond in the usual way to the extracellular acidosis which followed the rubidium load. Since renal excretion of acid constitutes the only mechanism by which animals normally regenerate their plasma bicarbonate after it has been lowered by acidosis, this failure of renal response, if maintained, would tend to perpetuate the acidosis.

To explain the absence of any renal response in the acidotic rubidium-loaded rats, it might at first seem reasonable to implicate the very rapid excretion of potassium which resulted from the rubidium load. There is good evidence in man and dog that loading with potassium inhibits secretion of acid (10), but the absence of any change in acid excretion in the potassium-loaded animals of this study, as well as other data in the literature (7, 9), suggest that this phenomenon is not readily demonstrable in the rat. In any event, recent studies have shown that possible hydrogen-potassium competition cannot explain the behavior of rubidium-substituted rats, because there is no increased acid excretion even when urinary potassium and rubidium are very low (11). Further work will be required to clarify this question.

SUMMARY AND CONCLUSIONS

Electrolyte and acid-base balance studies were carried out on normal rats fed 40 mEq. per Kg. of RbCl over a period of two days and the results compared with those resulting from an equivalent load of KCl.

Loading with KCl resulted in little or no change in electrolyte balance or plasma CO_2 content. By contrast, loading with RbCl resulted in cellular accumulation of approximately 29 mEq. per Kg. of rubidium and simultaneous renal loss of approximately 28 mEq. per Kg. of potassium and 5 mEq. per Kg. of sodium. When corrected for the negative balance during the control period, net potassium loss during rubidium loading amounted to approximately 22 mEq. per Kg. These renal losses seemed largely to represent the displacement of intracellular cation by rubidium.

The administration of rubidium also produced a severe extracellular metabolic acidosis which was not accompanied by increased urinary excretion of acid. There was, instead, a slight reduction in ammonium excretion. Thus it would appear that defective renal compensation plays an important role in the maintenance of acidosis in rubidium-substituted rats.

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THE FATTY ACID PATTERNS OF PLASMA LIPIDS DURING ALIMENTARY LIPEMIA *

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The total amount of fatty acid contained in the blood stream at any moment is considerably less than the amount of fat that might be taken in a single feeding. Therefore, during the period of alimentary lipemia some of the lipids in blood would be expected to acquire the composition of dietary fat.

To illustrate, the blood plasma of a 70 Kg. man contains about 50 mEq. of fatty acids (approximately 25 mEq. in phospholipids, 12 mEq. in cholesterol esters, 10 mEq. in triglycerides and 3 mEq. in nonesterified fatty acids), whereas a meal containing 3 ounces of fat provides 300 mEq. The amount of fatty acid entering the blood during a period of a few hours after such a feeding thus is about six times the total amount in circulation and, since the concentration of fatty acid in plasma remains approximately constant during absorption, the fatty acids of plasma must turn over on the average about six times. The actual turnover of transport lipids must be much greater than this since only a small part of the plasma lipid participates directly in absorption. Practically all of the newly absorbed fat enters the blood as triglyceride (1, 2). If dietary fat were simply mixed with this small pool and then transported to the tissues, the pool would turn over about 30 times and the composition of plasma triglyceride would become identical with that of dietary fat during the period of absorption.

Some evidence of this expected effect was obtained by Wilson and Hanner (3) who fed large doses of cream or cod liver oil to children and observed that the iodine number of the increment of blood lipids during the lipemic phase became approximately equal to the iodine number of the fed fat. It is also known that the composition of adipose tissue in various animals (4) and man (5) can be changed by feeding diets containing large amounts of unsaturated acids; therefore, there can be little doubt that the blood stream must transport a substantial part of the dietary fatty acid from gut to depot without modification.

On the other hand, a number of studies in the older literature [reviewed by Bloor (6)] showed that the iodine number and melting point of fat in thoracic duct lymph can differ significantly from the corresponding values for the fat that had been fed, and therefore that the fat delivered into the blood need not have the same fatty acid pattern as the dietary mixture. More recently Fernandes, Van de Kamer and Weijers (7) in studying a child with chylothorax found that the pattern of fatty acids in the chyle was influenced by the kind of fat fed during the preceding week, but significant differences between chyle and dietary fat always remained.

In the present study it was found that absorption of 100 Gm. of corn oil, coconut oil or butter caused remarkably little change in the fatty acid patterns of chylomicra in plasma or in the patterns of other plasma fractions. These results and the results of other studies of the absorptive process must be distinguished clearly from the effects of feeding some distinctive fat over a period of many weeks (8-11). In the present case one is concerned with a transport process; in the latter, with a slow change in composition of tissue lipids caused by repeated feedings and the manifestation of this change in the plasma of fasting subjects.

METHODS

Chylomicra were separated from plasma by flotation into a supernatant layer of saline (100,000 G × 30 minutes) (12).

Nonesterified fatty acids were extracted into heptane with the heptane-acid-isopropyl alcohol system previously described (13) and then were transferred into 90 per cent ethanol-water by titration of a two-phase heptane

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extract, ethanol-water system with 0.02 N NaOH. After extraction of the alkaline ethanol-water phase with petroleum ether to remove contaminating esters, the nonesterified fatty acids were methylated for chromatography.

Plasma lipids were extracted with ether-ethanol; the extract was evaporated to dryness and the cholesterol ester plus triglyceride fraction separated from the phospholipids by extraction of the residue with acetone. The esterified fatty acids were saponified in 10 per cent methanolic KOH, extracted after removal of nonsaponifiable material and methylated with methanolic HCl.

The methyl esters were stored as solutions in petroleum ether (B. P., 40 to 60° C.) in the cold until analyzed. Separations were carried out using two types of stationary phase: *a*) Apiezon L vacuum stopcock grease at 197° C. (14); and *b*) a polymer of ethylene glycol and adipic acid at 175° C. (15-17). In the earlier work, the gas-density balance (18) was used as the vapor detector, total loads of ester separated being approximately 3 mg. At this level of sensitivity large samples of plasma were required (50 to 75 ml.) particularly when dealing with the nonesterified fatty acid fractions. The introduction of the much more sensitive argon ionization detector (19, 20) allowed a total column load of 100 μg. or less to be used, so that more detailed physiological studies were possible with much smaller blood samples. Peak areas were measured by triangulation and analyses were usually carried out at two or more levels in order to determine major and minor components accurately. The analytical figures given refer to percentage by weight of a given component in the acids of chain length C_8 and C_{20}.

The course of lipemia in some experiments was followed by determination of triglyceride by the method of Van Handel and Zilversmit (21).

EXPERIMENTAL PROCEDURE

For control data samples of plasma were obtained from two groups of human subjects: male medical students and a somewhat older group of male and female laboratory personnel. All subjects appeared healthy and of usual dietary habits. At the time of testing all subjects were at least 12 hours post prandial. The analyses of animal plasma fractions were made on specimens obtained from fasting animals maintained on commercial feeds.

Fasting male medical students from the blood donor list served as subjects for the feeding experiments. After an initial sample of blood had been taken, each subject was fed 100 Gm. of test fat (corn oil, coconut oil or butter) with no other food. Thereafter they were bled three times: at one-half, one, and two and one-half hours (short-term studies) or at three, six and nine hours (longer term). During the period of observation the subjects remained ambulatory and were permitted water, *ad libitum*, but nothing else by mouth.

Blood was taken into heparinized flasks and centrifuged promptly to remove cells. Because of the relatively low sensitivity of the chromatographic detector available in 1957 it was necessary to take about 100 ml. of blood in each sample. The more recent analyses have required only 10 ml. of blood. Fortunately these later studies have given essentially the same results as the earlier experiments, which suggests that removal of 300 to 400 ml. of blood does not distort the fatty acid patterns, and so the data from the earlier and the more recent experiments are considered together.

TREATMENT OF THE DATA

The wide variety of fatty acids now shown to occur in animal fats makes the familiar terminology inadequate and the systematic chemical nomenclature too cumbersome for tabulation of analytical results. We have found it convenient to identify fatty acids by a dual symbol: the first figure denoting chain length, and the second, the number of double bonds (*e.g.*, 16:0 for palmitic acid, 18:1 for oleic acid and 18:2 for linoleic acid). The symbol can be elaborated further to show whether the chain is branched or straight (*e.g.*, branched 15:0) and positional isomers can be distinguished by suffixes (*e.g.*, $18:1^{\omega-10}$). However, even with this simplified tabulation it is difficult to compare mixtures having the same major but different minor components. We have explored the possibility of using statistical methods for measuring the similarity of different patterns but the calculations have not been helpful to date because the relative importance of different components is not yet defined.

The "double bond index" (DBI) shown in the tables is a measure of unsaturation, calculated from the formula, $DBI = \Sigma(n_i w_i / m_i)$ (where n_i is the number of double bonds in the fatty acid i, m_i is the molecular weight and w_i the percentage by weight of the fatty acid in the given mixture). The factors n_i/m_i, given in Table I, were multiplied by the corresponding percentages obtained from the analysis and the products were summed to give the number of double bonds per unit weight of mixed fatty acids.

RESULTS

Fasting subjects

Tables II and III give the fatty acid composition of the various plasma lipids isolated from both humans and animals. The category "18:1 isomers" refers to isomers of oleic acid that consist mainly of trans-11:12-octadecanoic acid (vaccenic) (*22*); 20:3 refers to an acid whose structure has been partially elucidated by degradation; and 18–22:? refers to a group of unsaturated acids whose structure has yet to be determined.

Short experiments

Table IV shows the compositions of the three different dietary fats and the fatty acid patterns of nonesterified fatty acids before and up to two and one-half hours after the feedings, each datum

TABLE I

Numerical factors for calculation of double bond index (DBI)

Acid	12:1	14:1	16:1	18:1	18:2	18:3	20:?
$\frac{n_i}{m_i}$	0.505	0.442	0.394	0.354	0.714	1.080	1.315

in the table being the average of two tests with different subjects. There was essentially no change in the patterns except for a rise in lauric (12:0) and myristic (14:0) acids after ingestion of coconut oil. Statistical analyses verified the significance of these rises and also indicated that none of the other variations in pattern (including fluctuations in trace components not shown in the table) had any significant relation to the pattern of dietary load. The double bond indices (which measure net unsaturation) remained essentially constant.

Longer experiments

The experiments running up to nine hours did show a number of significant changes (Tables V and VI), but of relatively minor degree. For instance, the chief components of corn oil feedings, the unsaturated C_{18} acids, comprised 89 per cent of the total load [oleic (18:1) and its isomers accounted for 34 per cent, and linoleic (18:2) for about 55 per cent], yet during absorption, the proportion of 18:2 in each plasma fraction rose only moderately, remaining well below the dietary

TABLE II

*Composition of plasma lipids in human subjects**

Component	NEFA†	Chylomicra	Phospholipid	Triglycerides-plus-cholesterol-esters
No. of subjects	18	6	6	6
	%	%	%	%
Saturated				
12:0	0.3 ± 0.4	1.9 ± 1.5	0.1 ± 0.1	0 ± 0
14:0	1.7 ± 0.9	3.7 ± 1.4	1.1 ± 0.8	1.0 ± 0.4
15:0		0.8 ± 0.8	0.5 ± 0.5	0.2 ± 0.2
16:0	23.2 ± 2.8	24.2 ± 4.7	33.1 ± 3.8	21.4 ± 2.2
17:0		0.4 ± 0.4	0.5 ± 0.5	0.5 ± 0.3
18:0	12.9 ± 2.6	10.4 ± 1.7	12.8 ± 3.3	4.2 ± 1.2
20:0			0.3 ± 0.4	
Monounsaturated				
12:1		0.9 ± 1.0	0.4 ± 0.1	0 ± 0.1
14:1	0.6 ± 1.0	1.4 ± 1.0	0.5 ± 0.5	0.4 ± 0.3
16:1	2.4 ± 0.9	3.0 ± 1.0	2.5 ± 0.7	3.4 ± 1.0
18:1$^{9-10}$	28.9 ± 5.0	21.8 ± 4.8	18.6 ± 4.7	26.4 ± 5.5
18:1$^{\text{isomers}}$	3.7 ± 1.3			
Diunsaturated				
18:2	14.5 ± 4.1	8.7 ± 1.2	16.0 ± 6.5	33.5 ± 5.5
Polyunsaturated				
18:3		0.3 ± 0.3	0.5 ± 0.6	0.5 ± 0.5
20:3	1.3 ± 1.5	2.0 ± 2.0	1.8 ± 0.6	1.0 ± 0.5
20:4	4.7 ± 2.6	0.5 ± 0.5	4.3 ± 2.1	4.2 ± 2.0
18 → 22:?		8.0 ± 8.0	4.1 ± 4.0	2.0 ± 2.0
Sum	94.2	88.0	97.1	98.7
Double bond index	28.0 ± 5.7	32.5 ± 11.4	33.3 ± 3.5	44.1 ± 2.8

* Values given in the main part of the table are the mean and standard deviation of percentage by weight for each component. The sum is less than 100 per cent in each case because inconstant trace amounts of unusual acids, detected in individual analyses, were omitted from the final tabulations. The double bond index, defined in the text, measures degree of unsaturation.

† Nonesterified fatty acids.

TABLE III

*Composition of plasma lipid fractions in various animals**

Component	Sheep			Cow			Pig			Horse			Chicken		
	NEFA†	PL‡	TG+CE§	NEFA	PL	TG+CE	NEFA	PL	TG+CE	NEFA	PL	TG+CE	NEFA	PL	TG+CE
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Saturated															
12:0			0.3			0.6	1.1	0.4		0.3	0.2	0.1	0.2	0.5	0.5
14:0	1.5	1.8	1.1	1.6	1.4	1.1	1.0	0.9	0.3	1.1		0.7	0.9	1.2	0.9
15:0	0.4		0.6	0.7	0.3	0.6			0.2	0.3	0.4	0.3		0.7	0.2
16:0	20.6	22.9	17.4	23.4	27.1	15.2	16.5	19.8	14.8	16.1	17.7	16.2	20.7	21.2	18.1
17:0	1.3	0.7	0.7	0.9	0.6	1.5	1.4	1.8	1.8	0.6	0.4	0.6		0.6	0.4
18:0	18.3	22.4	13.8	18.6	20.1	7.3	22.2	21.3	15.3	18.5	23.5	6.7	21.2	26.2	9.0
Monounsaturated															
12:1															
14:1	0.4	0.3	0.2	0.4	0.3	0.6	0.6	0.9	0.2	0.5		0.3	0.9	0.7	0.8
16:1	1.8	0.6	2.3	3.0	1.6	3.3	1.6	1.8	1.4	1.4	0.9	1.6	2.0	2.8	4.0
18:1$^{9-10}$	42.9	25.6	28.0	33.7	23.0	11.6	22.6	25.0	26.7	17.2	14.4	17.0	24.7	19.2	30.2
18:1$^{\text{isomers}}$	3.5	2.4	2.4	4.0	3.0								1.5		
Diunsaturated															
18:2	5.3	13.6	24.3	5.9	14.9	43.4	16.7	19.2	27.5	37.4	41.3	54.0	16.3	15.3	24.7
Polyunsaturated															
18:3						2.0			0.8	1.9		1.2			0.5
20:3		1.2		1.0	1.2	1.2		1.7	0.8				1.5	1.8	1.8
20:4		4.6	3.0	1.1	3.6	4.8	9.6	6.1	7.0	1.1	0.7	0.5	8.0	7.0	3.6
18 → 22:?							1.9		1.6	2.3		0.6			
Sum	96.0	96.7	94.1	98.0	96.1	93.2	91.3	98.9	98.4	98.7	99.4	99.8	97.8	97.2	94.7
Double bond index	21.1	27.6	33.1	21.7	26.9	46.7	35.9	33.9	43.0	40.1	43.5	48.1	34.6	30.7	37.9

* Data obtained from fractionation and analysis of a single plasma specimen from each animal.
† Nonesterified fatty acids.
‡ Phospholipids.
§ Triglyceride plus cholesterol ester.

TABLE IV

NEFA patterns before and after feeding 100 Gm. of indicated fat†*

Component	Corn oil					Coconut oil					Butter				
	Oil	Fasting	½ hr.	1 hr.	2½ hrs.	Oil	Fasting	½ hr.	1 hr.	2½ hrs.	Butter	Fasting	½ hr.	1 hr.	2½ hrs.
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Saturated															
12:0		0.3		0.3	0.1	39.8	0.7	1.2	0.9	10.7	3.8	0.3	0.1	0.2	0.8
14:0		1.2	0.8	1.0	1.0	22.7	3.6	2.0	1.9	10.1	18.2	1.3	1.4	1.7	2.4
16:0	9.8	21.8	21.3	22.3	22.7	11.0	25.2	21.0	19.9	21.3	20.8	21.0	23.7	23.5	21.3
18:0	1.1	13.2	13.9	13.6	12.0	3.6	16.4	15.0	13.8	11.0	16.4	12.6	14.6	14.7	14.7
20:0															
Monounsaturated															
12:1		0.2		0.1	0.1		0.3		0.3						
14:1		0.2	0.1	0.3	0.3		0.2	1.6	3.8	2.7	1.0	1.1	0.3	0.8	1.0
16:1		2.2	1.0	1.8	1.3		2.1	2.4	2.0	1.6	1.8	2.1	2.3	1.6	1.3
18:1	33.9	32.0	29.5	28.2	32.0	9.2	35.6	33.6	36.5	29.1	24.2	34.6	32.7	31.2	26.9
Diunsaturated															
18:2	55.1	12.8	16.4	17.8	19.8	2.6	6.8	11.3	11.3	7.3	2.5	16.4	14.4	16.2	10.1
Polyunsaturated															
18:3															
18 → 22:?		12.2	7.9	7.6	6.4		4.0	2.8	5.2	2.0	1.0	6.0	6.3	7.5	6.2
Sum	99.9	96.1	90.9	93.0	95.7	88.9	94.9	90.9	95.6	95.8	89.7	95.4	95.8	97.4	84.7
Double bond index	51.3	37.6	33.0	33.6	34.6	5.1	23.8	25.3	30.4	20.0	12.8	33.2	31.2	33.5	25.8

* Nonesterified fatty acid.

† The component fatty acids are designated by a dual symbol giving chain length and number of double bonds. Values in the main part of the table are the percentages by weight of the indicated fatty acids, each datum being the average of two experimental values. The sum of each column is less than 100 per cent because trace amounts of unusual acids, detected in individual analyses, were omitted from the final tabulation. The double bond index measures degree of unsaturation.

TABLE V

Composition of plasma lipids at various times after ingestion of corn oil (100 Gm.) *

Component	Corn oil	Fasting				3 hrs. after loading				6 hrs. after loading				9 hrs. after loading			
		NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Saturated																	
12:0		0.1	1.8			0.3	0.7	0.1		0.6	0.1			0.5	1.5		
14:0		1.6	3.6	0.7	0.9	1.4	2.6	0.6	0.6	1.4	1.5	0.7	0.5	1.3	1.5	0.7	0.3
15:0		0.5	0.8	0.4	0.2	0.7		0.4			0.3	0.2	0.1	0.2		0.2	0.1
16:0	9.8	22.5	22.8	31.2	21.1	17.5	26.0	25.6	20.7	18.5	24.8	33.4	19.9	19.3	22.1	29.7	17.9
17:0		0.2	0.5	0.6	0.6	0.3	0.4	1.1	0.3	0.1	0.2	0.6	0.2	0.4	0.2	0.4	0.5
18:0	1.1	10.9	10.6	11.5	4.4	9.3	9.7	13.0	4.5	9.9	7.5	13.5	3.5	9.5	10.6	12.9	3.2
20:0							0.5										
Monounsaturated																	
12:1			0.6				0.4	0.1							0.6		
14:1		1.6	1.4	0.3	0.5	1.4	1.0	0.6	0.3		0.4	0.3	0.2	0.4	0.4	0.7	0.2
16:1		2.4	2.6	2.7	3.2	1.7	2.2	2.7	2.6	1.6	3.2	2.0	2.4	2.0	3.9	2.8	3.2
18:1	33.9	31.7	18.4	19.7	25.0	29.0	21.3	19.2	26.9	21.6	26.5	17.6	25.9	25.0	25.6	16.9	22.6
Diunsaturated																	
18:2	55.1	16.4	8.3	20.1	34.7	21.8	11.6	15.6	36.6	24.7	21.7	18.7	39.7	28.9	24.1	21.8	42.0
Polyunsaturated																	
18:3				0.5	0.4			0.7	0.7			0.1	0.3		0.2		0.6
20:3		1.3	1.9	1.9	1.2	3.6	0.7	1.6	0.8	5.0	1.0	1.6	2.2	2.0	0.2	1.9	0.7
20:4		4.3	0.3	5.4	3.8	4.6	0.2	4.3	3.7	2.6	1.0	3.6	2.1	4.8		4.4	2.4
18 → 22:?			12.8	1.8	1.3		19.3	5.3	1.7		3.3	4.5	0.9		5.2	2.9	3.0
Sum	99.9	93.5	86.4	96.8	97.3	91.6	96.6	90.9	99.4	86.0	91.8	96.8	97.9	94.3	96.1	95.3	96.7
Double bond index	51.3	31.9	34.1	35.0	43.8	37.9	43.9	34.8	45.7	35.9	33.3	33.4	45.7	39.4	35.6	35.1	48.0
Turbidity of plasma			±				+ to ++				+++ to ++++				++ to +++		
Plasma triglyceride (μMoles/L.)			847				933				1380				920		

* For explanation of symbols see Table IV. The datum for NEFA in each case is the average of two experimental values; all other data are the averages of three values. The turbidity was judged by inspection and ranked on a scale of 0 to ++++.

TABLE VI

Composition of plasma lipids at various times after ingestion of coconut oil (100 Gm.) *

Component	Coconut oil	Fasting				3 hrs. after loading				6 hrs. after loading				9 hrs. after loading			
		NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE	NEFA	Chylo	PL	TG+CE
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Saturated																	
10:0	6.7	0.2	0.3			0.7	2.3			0.5	1.1			0.4	2.3		
12:0	39.8	0.4	1.5			5.7	3.9	0.5		4.5	6.9	0.6		4.4	6.3	1.1	
14:0	22.7	2.3	3.4	0.9	0.9	3.7	3.7	1.1	1.4	3.6	5.9	0.9	1.0	4.3	5.1	1.6	1.2
15:0		0.5	0.2	0.2	0.1	0.5	0.4	0.1	0.2	0.1	1.3	0.2	0.2	0.1	0.7	0.1	0.2
16:0	11.0	24.0	25.6	34.7	21.5	22.1	19.7	32.4	20.3	22.1	24.2	33.8	17.8	22.2	19.2	34.8	20.2
17:0		0.8	0.1	0.1	0.2	0.2	0.1	0.5	0.2	0.3	0.5	0.3	0.3	0.2	0.4	0.3	0.4
18:0	3.6	11.7	10.1	14.2	4.0	12.0	7.6	15.1	3.7	12.2	5.5	14.2	2.9	12.8	7.1	16.2	3.5
20:0				0.3				0.2				0.3				0.5	
Monounsaturated																	
12:1			1.1			0.1	1.5	0.2			1.4	0.2			2.3	0.6	
14:1		1.2	1.3	0.7	0.3	1.1	1.5	0.3	0.3	1.1	3.1	0.3	0.2	0.4	3.1	0.5	0.3
16:1		2.4	2.9	2.3	3.6	2.1	2.9	2.2	3.9	2.0	3.8	2.8	4.4	2.3	2.5	2.7	3.3
18:1	9.2	33.2	25.1	17.4	27.7	28.3	23.9	13.9	29.1	28.4	16.5	16.0	30.1	32.2	18.6	13.0	27.1
Diunsaturated																	
18:2	2.6	17.0	8.5	10.3	30.5	15.7	9.1	10.5	31.2	16.3	6.5	12.4	33.5	15.7	9.9	9.9	30.3
Polyunsaturated																	
18:3			0.1	0.1	0.4		0.1		0.5			0.1	0.7			0.3	0.7
20:3		0.8	1.3	1.4	0.8	1.5	2.0	1.5	0.6	1.1	0.4	1.6	0.6	0.8	0.7	1.7	1.4
20:4		3.5	0.3	3.7	5.5	4.1	1.8	3.2	3.9	4.5	1.3	5.3	3.9	2.5		3.7	5.6
18→22:?			8.7	5.9	2.8		11.3	14.6	3.6		6.9	5.3	2.7		9.3	8.3	3.7
Sum	95.6	98.0	90.5	92.2	98.3	97.8	91.8	96.3	98.9	96.7	85.3	94.3	98.3	98.3	87.5	95.3	97.9
Double bond index	5.1	31.0	30.9	29.5	45.5	29.9	37.5	38.9	45.4	30.3	25.4	32.0	46.6	28.0	30.3	31.6	47.5
Turbidity of plasma			±				+				++ to +++				+ to ++		
Plasma triglyceride (μMoles/L.)			720				807				740				620		

* For explanation of symbols see Table IV. Data for NEFA are averages of four experimental values; all other data are the averages of three values. The turbidity was judged by inspection and ranked on a scale of 0 to ++++.

level, while 18:1 remained constant or fell so that the total C_{18} concentration remained essentially unchanged and the double bond index showed no consistent trend.

The stability of the patterns is illustrated in Figures 1 through 4, which show the fatty acid distributions classified by saturation and chain length. It will be seen in Figure 1 that chylomicra tended to maintain the pattern found in fasting samples, although the effect of the dietary load was perceptible. The pattern during absorption, considered as a mixture between dietary fat and the body lipids with the fasting pattern, suggests that dietary fat contributed at most only half of the oleic acid present in chylomicra (based on the elevation of 18:2) and, with respect to other components, a much smaller proportion.

The nonesterified fatty acids (Figure 2) also responded perceptibly to the dietary load, while

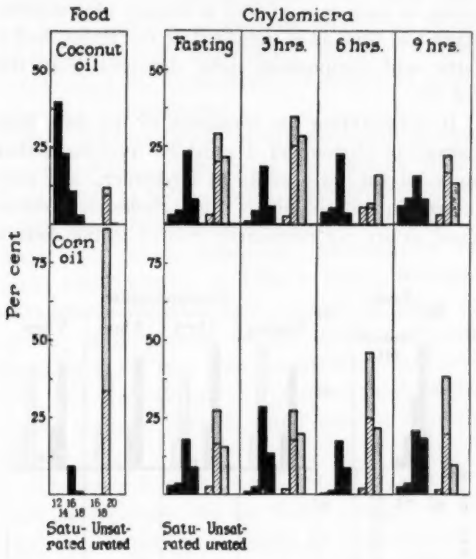

FIG. 1. PATTERNS OF FATTY ACIDS IN CHYLOMICRA AFTER FEEDING 100 GM. OF COCONUT OIL (UPPER ROW) OR CORN OIL (LOWER ROW)

The composition of dietary fat is shown on the left, followed by chylomicron patterns in blood samples obtained before feeding and at the indicated times subsequently. Saturated fatty acids are shown in solid black; monounsaturated acids are shown with crosshatching, and polyunsaturated, with stippling. The chain lengths, shown under the corn oil diagram, apply similarly to each of the other diagrams.

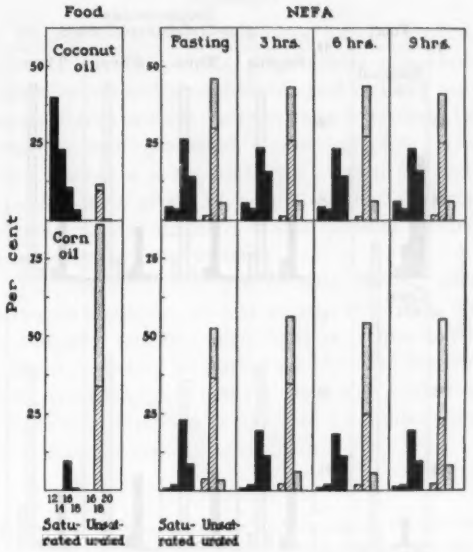

FIG. 2. NONESTERIFIED FATTY ACID (NEFA)

the patterns of triglyceride plus cholesterol esters (Figure 3) and phospholipids (Figure 4) were virtually unchanged.

DISCUSSION

Fasting subjects

As has been noted before (23), each class of lipid was found to have a distinctive composition with a relatively small spread of values for major and minor components within each class (Table II).

The high concentration of polyunsaturated acids found in chylomicra is of particular interest since chylomicra are known to contain less than 10 per cent of phospholipids (24, 25). It would seem, therefore, that either part of the triglyceride in chylomicra was highly unsaturated or the phospholipid component contained an exceptionally large amount of unsaturated acids. In either case some of the chylomicron lipid must have been derived from an endogenous source rich in polyunsaturated acids. In addition the chylomicra contained less linoleic acid than the other fractions.

Some of the present data can be compared with those obtained in a previous study of British subjects (23). The phospholipid composition is

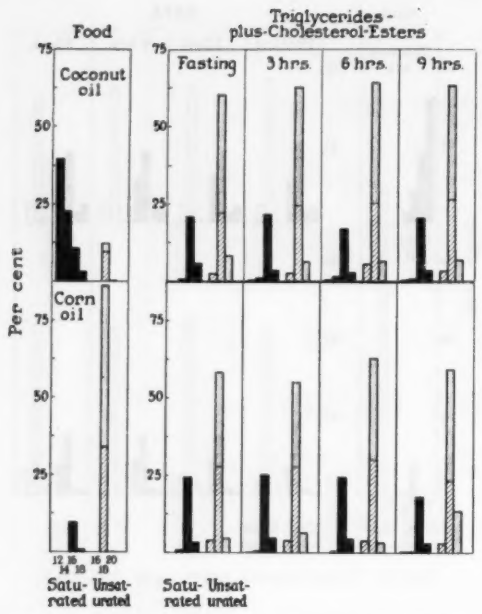

FIG. 3. TRIGLYCERIDES PLUS CHOLESTEROL ESTERS

similar in the two groups except that palmitic acid appears to be somewhat higher in the American group and arachidonic acid, lower. Larger differences are seen in the cholesterol ester plus triglyceride fraction; the American group had a much higher linoleic level (33.5 ± 5.5 per cent) than the British (12.4 per cent), while the British group showed a somewhat higher oleic acid level (34.7 per cent) than the Americans (26.4 ± 5.5 per cent). Presumably these differences were due to higher cholesterol/triglyceride ratios in the American subjects.

Table III shows the fatty acid compositions of nonesterified fatty acids, phospholipds and cholesterol esters plus triglyceride in plasma of sheep cow, pig, horse and chicken. The three lipid fractions appeared to have fairly consistent patterns in all the animals studied; this similarity is in striking contrast to the wide differences in kind of food eaten by the various species.

Lipemia

The degree of alimentary lipemia depends both on the kind of fat fed (26) and on the dose. Moderate loads, such as might be eaten in a normal meal, usually cause little or no rise in total lipid concentration of plasma (27) although they are reflected in chylomicronemia and turbidity during the period of absorption (28). To obtain a substantial rise in concentration of plasma fatty acids one must feed a large amount of fat, such as 3.5 Gm. per Kg. (29). In the experiment of Wilson and Hanner (3) children were fed even larger doses, up to 5.6 Gm. per Kg. Changes in plasma fatty acid pattern did occur under these conditions, but in interpreting the results one must note the unphysiological size of the load.

With the more moderate feedings used in the present experiments, the subjects in the corn oil group showed a slight rise in plasma triglyceride concentration while those in the coconut oil group showed no significant change; all subjects, however, showed definite turbidity of plasma during absorption, usually maximal at six hours. Centrifugation of the turbid plasma under a layer of saline in each case yielded a creamy chylomicron layer and this, as is indicated in the tables, had a fatty acid composition quite different from the fed fat.

In interpreting the constancy of the fatty acid pattern of chylomicra it must be recognized that some of the fatty acids in chylomicra are contained in phospholipids and cholesterol esters (and so are not necessarily related to the dietary

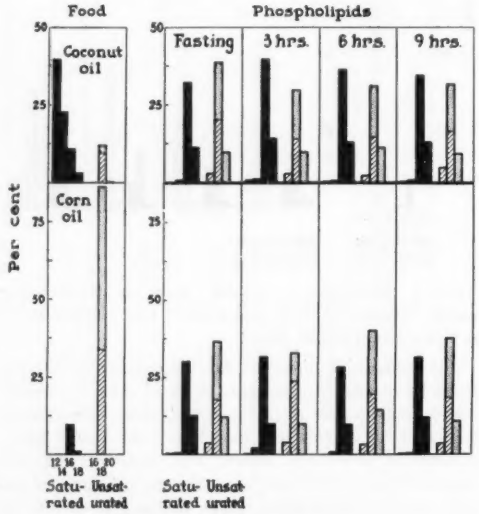

FIG. 4. PHOSPHOLIPIDS

load), and also that the flotation layer obtained from the upper part of the saline phase might be contaminated with nonchylomicron lipids. These explanations, however, fail to account for the discrepancy between chylomicra and fed fat. It is unlikely that the phospholipids and cholesterol esters in chylomicra could have contributed more than 10 per cent of the fatty acids (24, 25) and the composition of the chylomicron fraction was very far from what one would obtain by a mixture of nine parts of dietary fat with one part of phospholipid or any other fraction of the subnatant plasma. It also seems unlikely that any contamination of the flotation layer with nonchylomicron lipids could have been great enough to predominate over the chylomicron lipids; centrifugation of plasma from fasting subjects yielded only traces of lipids, while at the peak of lipemia, the same procedure yielded a heavy cream. Finally, the constancy in composition of the triglyceride plus cholesterol ester fraction showed that there could not have been any appreciable proportion of unmodified dietary triglyceride in this fraction (which includes chylomicron triglyceride) at the time of any observation.

Two different kinds of process might account for the relative stability of the various plasma fatty acid patterns during absorption. There might be, as Bloor suggested (6), a conversion of fatty acids during digestion. Alternatively, the fatty acid patterns of plasma lipids, including chylomicra, might be held constant by rapid recycling of fatty acids between blood and tissue pools, as is the case with nonesterified fatty acids (12, 30). Work now in progress in this laboratory indicates that the latter alternative is the correct one.

It is also of interest to note that the nonesterified fatty acid pattern did not follow changes in the chylomicron pattern as might be expected if the clearing of lipemia plasma were simply lipolytic and the nonesterified fatty acids were products. This result is in agreement with the experiments of Fredrickson, McCollester and Ono (31) who showed that not more than one molecule in 10 of the nonesterified fatty acids was derived from labeled chylomicra when these were infused into dogs at rates comparable to that of normal absorption.

SUMMARY

Plasma lipids were fractionated into chylomicra, nonesterified fatty acids, triglycerides plus cholesterol esters and phospholipids, and the fatty acid compositions of these fractions were determined by analysis with gas-liquid chromatography. The data showed a fairly consistent pattern for each fraction in a group of normal fasting subjects. A few analyses made on animal plasmas yielded essentially the same patterns.

Plasmas taken during alimentary lipemia were analyzed similarly. It was found that neither the chylomicra, nor any other fraction, acquired the pattern of dietary fat during the period of lipemia. This result suggests that the fatty acid pattern of chylomicra in plasma is stabilized by equilibration with a larger pool of tissue lipids.

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THE REDUCTION OF METHEMOGLOBIN IN HUMAN ERYTHROCYTES INCUBATED WITH PURINE NUCLEOSIDES * †

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This study is concerned with the effect of purine nucleosides and related compounds on the reduction of methemoglobin to hemoglobin in human erythrocytes *in vitro*. Other investigators have reported that mammalian erythrocytes can reduce methemoglobin to hemoglobin when the cells are incubated with glucose (2), lactate (3), glyceraldehyde, fructose (4), fumaric acid, malic acid (5), mannose, galactose (6), formaldehyde (7) and other aliphatic and aromatic aldehydes (8). Previous studies in this and other laboratories have demonstrated that normal human erythrocytes can metabolize the pentose moiety of certain purine nucleosides to lactic acid *in vitro* (9, 10). This metabolic activity is associated with enhanced resistance to osmotic stress (11, 12), increased organic phosphate esters (11), retention of potassium (11) and extrusion of sodium (13), maintenance of reduced glutathione (14, 15) and some prolongation of viability of erythrocytes stored *in vitro* (11, 16, 17).

In the present investigation it was observed that normal human erythrocytes in which 60 to 80 per cent of the hemoglobin was chemically oxidized *in vitro* could reduce a considerable portion of the methemoglobin to hemoglobin when incubated with certain purine nucleosides and sugars. This effect was associated with active metabolism of the compounds by the cells. The metabolism of these compounds and their effects on the reduction of methemoglobin in erythrocytes obtained from a patient with congenital methemoglobinemia were also studied.

MATERIALS AND METHODS

Whole blood was obtained from normal adults and was anticoagulated with about 1 mg. dry heparin per 10 ml. of blood. The volume of packed erythrocytes was determined by the method of Wintrobe (18). A portion of the blood was incubated at 37° C. with occasional mixing, after the addition of 0.6 to 0.9 mg. of sodium nitrite per ml. of packed erythrocytes. The sodium nitrite was dissolved in 1 ml. of isotonic sodium chloride solution. After 40 minutes' incubation, the treated blood was centrifuged at 1,500 G for seven minutes and the erythrocytes were washed three times with about three volumes of 0.9 per cent sodium chloride solution; the final centrifugation was carried out in a Servall Refrigerated Angle Centrifuge for 10 minutes at 1,900 G and at 4° C. (SS-1 rotor). A 25 per cent suspension of the treated and washed erythrocytes in isotonic sodium chloride solution was prepared and the actual volume of packed erythrocytes was determined. This procedure resulted in the conversion of 60 to 80 per cent of the hemoglobin to methemoglobin. Four ml. portions of the erythrocyte suspension were added to 25 ml. Erlenmeyer flasks containing 2 ml. of isotonic sodium chloride-sodium phosphate buffer solution (equal volumes of 0.9 per cent sodium chloride solution and isotonic sodium phosphate buffer, pH 7.3) in which were dissolved the compounds to be studied. The pH of these final suspensions was 7.3 to 7.4. The flasks were stoppered loosely with cotton plugs and incubated at 37° C. in a Dubnoff metabolic shaker-incubator oscillating 92 cycles per minute. In experiments where hemolysates were used, 50 per cent suspensions of treated and washed erythrocytes were prepared and the erythrocytes were hemolyzed by twice freezing and thawing rapidly. Two ml. of the hemolysate was added to flasks containing 3 ml. of distilled water, 1 ml. of isotonic sodium phosphate buffer, pH 7.3, and the dissolved compounds.

At intervals during the incubation, 0.4 ml. portions of each suspension were transferred to 10 ml. of 0.0167 M phosphate buffer, pH 6.6, and the methemoglobin concentration was determined by the method of Evelyn and Malloy (19), adapted for the Coleman Junior Spectrophotometer. The amount of methemoglobin was expressed as the per cent of the total hemoglobin pigment. The methemoglobin concentrations of the whole blood prior to treatment with sodium nitrite and of the erythrocyte suspensions or hemolysates prior to incubation were also determined. Duplicate samples assayed by this

* This work was presented in part at the Eastern Section Meeting of the American Federation for Clinical Research at Boston, Mass., December 12, 1958 (1).

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spectrophotometric method agreed within ± 1.5 per cent. In a few experiments, the methemoglobin concentrations were also estimated from the difference between the total hemoglobin content, determined by comparison with a cyanmethemoglobin standard (20), and the hemoglobin content determined by the oxygen binding capacity of the erythrocyte suspensions, determined in the laboratory of Dr. Charles W. Frank. The spectrophotometric and the gasometric methods agreed within ± 4.5 per cent. The spectrophotometric method described recently by Mills and Randall (21) was employed in some experiments to permit the calculation of both methemoglobin and choleglobin concentrations. The methemoglobin concentrations by this method and by the method of Evelyn and Malloy agreed within ± 2 per cent. Significant choleglobin concentrations were not detected in erythrocyte suspensions during 22 hours of incubation with the various nucleoside and carbohydrate substrates and only 1.8 per cent was present in a suspension incubated with ascorbic acid.

Penicillin G and streptomycin sulfate, 1 mg. of each, were added to the incubation flasks in most of the experiments detailed in this paper. Even after 22 hours of incubation there was no bacterial contamination as evidenced by the absence of growth when aliquots of the suspensions were cultured on blood agar and in infusion broth. Identical results in the reduction of methemoglobin to hemoglobin were observed, however, in experiments performed with and without added antibiotics.

Lactic acid determinations were performed by the method of Barker and Summerson (22) on clear filtrates of perchloric acid extracts prepared by adding one volume of each erythrocyte suspension to six volumes of cold 2 per cent perchloric acid after the suspensions had been incubated for six hours.

Determinations of the pH of the erythrocyte suspensions after 22 hours of incubation were made with a Beckman Model G glass electrode pH meter. The pH of the control suspensions ranged from 7.2 to 7.5, but the pH of the suspensions incubated with the various compounds did not differ from that of the control by more than ± 0.1 pH unit. These changes in the pH could not be correlated with the effect of the compounds on the reduction of methemoglobin to hemoglobin.

The degree of spontaneous hemolysis that occurred during incubation was determined by adding 0.2 ml. aliquots of each suspension to 2 ml. of isotonic sodium chloride solution and 2 ml. of distilled water. The amount of hemoglobin was determined, after centrifugation and dilution of 1 ml. of the supernatant solution with 4 ml. of distilled water, by measuring the optical density at 540 mμ in a Beckman DU spectrophotometer. The per cent spontaneous hemolysis was then calculated by relating the optical density of the isotonic sodium chloride solution sample to the distilled water sample whose hemolysis was taken as 100 per cent. Spontaneous hemolysis, even after 22 hours of incubation, did not exceed 4 per cent.

Most of the compounds used in these experiments were obtained commercially. The 2,6-diaminopurine riboside (2,6-diamino-9-β-D-ribofuranosyl purine) and the adenine glucoside (9-β-D-glucopyranosyl adenine) were synthesized and provided by Dr. Bertram A. Lowy. Samples of purine riboside (9-β-D-ribofuranosyl purine) and adenosine-1-N-oxide were generously provided by Dr. George B. Brown. Mr. David Schwarz of Schwarz Laboratories supplied the adenosine-5′-phosphoramide. Dr. Henry M. Kissman of the Lederle Laboratories Division of the American Cyanamid Company kindly supplied the Puromycin Hydrochloride® (6-dimethylamino-9 (3′-p-methoxy-L-phenylalanyl amino-β-D-ribofuranosyl) purine), dimethyl-adenosine (6-dimethyl-amino-9-β-D-ribofuranosyl purine), amino-adenosine (6-amino-9 (3′-amino-3′-deoxy-β-D-ribofuranosyl) purine), aminonucleoside (6-dimethyl-amino-9 (3′-amino-3′-deoxy-β-D-ribofuranosyl) purine) and aminoribose (3-amino-3-deoxyribose) hydrochloride. The concentrations of the compounds were expressed as micromoles per milliliter of packed erythrocytes and were contained in a final volume of 6 ml.

Case history. The patient (E. D., Hospital No. 30216) was a 30 year old Puerto Rican woman who was seen in the Prenatal Clinic of the Bronx Municipal Hospital Center during the eighth month of her fifth pregnancy in November, 1957. At that time cyanosis of the hands, lips and vaginal mucosa was noted. She reported frequent headaches, slight exertional dyspnea and easy fatigability. Except for these findings, physical examination was entirely normal with a normal intrauterine pregnancy. The hemoglobin concentration was 11.6 Gm. per cent, the hematocrit 40 per cent and the erythrocytes appeared slightly hypochromic on the stained blood smear. The reticulocyte count ranged from 0.8 to 1.5 per cent. The white blood cell count, differential count, urine analysis, serology, roentgenogram of the chest and liver chemistries were within normal limits. Spectrophotometric examination of hemolysates prepared from whole blood and from washed erythrocytes obtained from a sample of the patient's blood revealed the absorption spectrum of methemoglobin as characterized by a peak at 630 mμ that disappeared on the addition of sodium cyanide. On repeated examinations the concentration of methemoglobin ranged between 23 and 26 per cent, but on one occasion was 31 per cent. One hour after the intravenous administration of methylene blue, 1 mg. per Kg., the patient's mucous membranes were of a normal pink color and the concentration of methemoglobin was 1 per cent. The methemoglobin concentration was maintained at a level of 9 to 13 per cent by the daily oral administration of 500 mg. of ascorbic acid and was accompanied by relief of her symptoms. That the abnormal hemoglobin in the erythrocytes of this patient was actually normal methemoglobin and not hemoglobin M was further established by spectrophotometric analysis of a hemolysate treated with potassium ferricyanide (23) and by starch block electrophoresis of a hemolysate kindly performed by Miss Margaret Pease through the cooperation of Dr. Park S. Gerald.

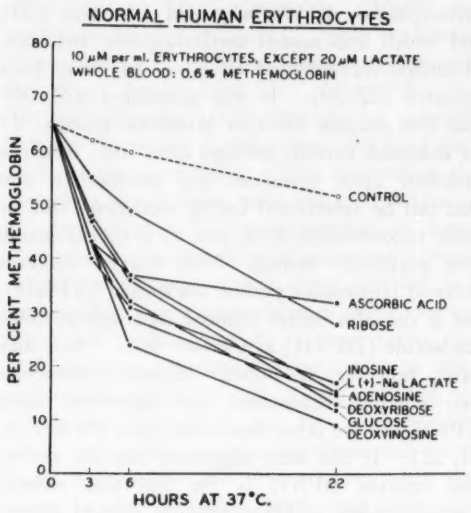

FIG. 1. EFFECT OF INCUBATION WITH VARIOUS COMPOUNDS ON THE REDUCTION OF METHEMOGLOBIN IN NORMAL HUMAN ERYTHROCYTES

RESULTS AND DISCUSSION

Normal human erythrocytes

Data from a typical experiment are presented in Figure 1 and Table I, Column A. Incubation with 10 μMoles per ml. of erythrocytes of inosine, adenosine, deoxyribose, glucose and deoxyinosine, as well as L (+)-sodium lactate, present in twice the molar concentration of the other compounds, resulted in the reduction of methemoglobin to hemoglobin at a much greater rate than occurred in the control erythrocyte suspension incubated without substrate. After six hours of incubation, almost identical amounts of lactic acid were present in the perchloric acid extracts of the erythrocyte suspensions incubated with adenosine, inosine and deoxyinosine, while perhaps slightly more was found in the suspension incubated with glucose. Although about 30 per cent less lactic acid had been formed after six hours of incubation with deoxyribose, the effect on methemoglobin conversion was only slightly less than that of the purine nucleosides and glucose. The extent of reduction of methemoglobin by deoxyribose was indistinguishable from that of the purine nucleosides and glucose after 22 hours. Incubation with ribose resulted in slower reduction of methemoglobin to hemoglobin than did incubation with the other compounds, and after six hours only about one quarter as much lactic acid was present as in the suspensions incubated with the purine nucleosides and glucose. L (+)-sodium lactate, 20 μMoles per ml. of erythrocytes, affected the reduction of methemoglobin at about the same rate as deoxyribose, 10 μMoles per ml. After 22 hours, however, the effect of lactate was essentially the same as that of the purine nucleosides and sugars. About 30 per cent of the lactate added had disappeared from the total erythrocyte suspension after six hours of incubation. Although initially ascorbic acid had almost the same effect as the various purine nucleosides, glucose, deoxyribose and lactate, this compound was the least effective after 22 hours of incubation. From the data presented in Table II it is apparent that incubation with guanosine, 2,6-diaminopurine riboside, xanthosine, deoxyadenosine and deoxyguanosine also had a marked effect on the reduction of methemoglobin to hemoglobin. In every instance, after 22 hours of incubation, the effect of the purine deoxyriboside was slightly greater than the effect of the corresponding purine riboside. The reason for this observed difference in effect is obscure. Although incubation with fructose and galactose promoted the reduction of methemoglobin to hemoglobin, these hexoses were less effective than an equimolar amount of glucose. A significant effect from incubation with fumaric acid could be demonstrated only

TABLE I

Lactic acid present after six hours of incubation of A) normal human erythrocytes and B) erythrocytes of congenital methemoglobinemia with purine nucleosides and sugars

Compound	A Normal human erythrocytes	B Erythrocytes of congenital methemoglobinemia
	*μMoles/ml.**	*μMoles/ml.**
Glucose	1.09	1.51
Inosine	1.02	1.18
Adenosine	0.99	1.18
Deoxyinosine	0.96	1.07
Deoxyribose	0.69	0.73
L (+)-sodium lactate	0.68	0.87
Ribose	0.25	0.31
Ascorbic acid	0	0

* Micromoles of lactic acid per micromole of substrate added per milliliter of erythrocytes, corrected for control.

TABLE II

The effect of purine nucleosides, sugars and related compounds on the reduction of methemoglobin in normal human erythrocytes

Compound	Concentration*	Initial methemoglobin	Methemoglobin after incubation for:		
			3 hrs.	6 hrs.	22 hrs.
		%	%	%	%
Control		70	69	67	62
Adenosine	8		47	38	24
Deoxyadenosine	8		50	31	18
Control		65	65	62	50
Inosine	10		42	28	13
Guanosine	10		44	30	12
Deoxyguanosine	10		45	24	5
Control		62	59	60	47
Adenosine	8		43	32	12
2, 6-DAPR†	8		44	31	10
Xanthosine	8		50	40	13
Control		77	73	73	63
Glucose	9		57	43	20
Fructose	9		68	56	32
Galactose	9		69	63	37
Fumaric acid	19		68	64	48
Control		65	65	63	56
Adenosine	10		42	29	15
Aminoadenosine	10		63	60	39

* Concentration in micromoles per milliliter of erythrocytes.

† 2,6-Diaminopurine riboside.

when this compound was present in twice the molar concentration of the other sugars. These experiments demonstrated that the compounds effective in promoting the reduction of methemoglobin to hemoglobin included purine ribosides (adenosine, guanosine, inosine, 2,6-diaminopurine riboside and xanthosine), purine deoxyribosides (deoxyadenosine, deoxyguanosine and deoxyinosine), hexoses (glucose, galactose and fructose), pentoses (ribose and deoxyribose), fumaric acid, L (+)-sodium lactate and ascorbic acid.

Although the precise mechanisms by which methemoglobin in intact erythrocytes is reduced to hemoglobin are still unknown, there is considerable evidence that one or more enzyme systems are involved. The normal process of methemoglobin reduction is dependent upon the integrity of the erythrocyte (2, 24), is associated with carbohydrate metabolism (4, 25) and requires the regeneration of reduced pyridine nucleotides (4, 26). Recently, partial purification, identification and characterization of an enzyme from human erythrocytes whose existence was implied by the investigations of Warburg and Christian (24) and which was named methemoglobin reductase (Hämiglobinreduktase) by Kiese (4) have been reported (27–30). It was postulated (28, 30) that this enzyme has two prosthetic groups: *1*) an unknown carrier, perhaps ionic iron, which is detached upon hemolysis and purification and that can be substituted for by methylene blue or other autoxidizable dyes, and *2*) a tightly bound iron porphyrin moiety. This enzyme requires reduced triphosphopyridine nucleotide (TPNH), but it can also utilize reduced diphosphopyridine nucleotide (DPNH) at a slower rate. There may exist, however, in normal human erythrocytes two separate reductases, one dependent upon TPNH and the other dependent upon DPNH (4, 31, 32). It has been suggested that the system that utilizes DPNH is the one that reduces methemoglobin to hemoglobin in normal human erythrocytes in the absence of methylene blue (32).

Metabolic pathways for the regeneration of either of the two reduced pyridine nucleotides are present within normal human erythrocytes. For example, reduction of triphosphopyridine nucleotide (TPN) to TPNH can occur via the hexose-monophosphate shunt upon metabolism of glucose-6-phosphate to 6-phosphogluconate and 6-phosphogluconic acid to ribulose-5-phosphate. Reduction of diphosphopyridine nucleotide (DPN) to DPNH can occur by way of the Embden-Meyerhof pathway upon the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and by the oxidation of lactate to pyruvate.

The results of the experiments described here may be explained by the metabolism of the ribose or deoxyribose portion of the purine nucleosides by way of the hexose-monophosphate shunt and the Embden-Meyerhof pathways with the regeneration of reduced pyridine nucleotides. Phosphorolytic cleavage of inosine or guanosine by erythrocyte purine nucleoside phosphorylase (33–35) results in the formation of ribose-1-phosphate that can be isomerized to ribose-5-phosphate. This latter compound can enter the hexose-monophosphate shunt with the formation of hexose phosphate and triose phosphate (36) which may be metabolized further by way of the Embden-Meyerhof pathway. Adenosine is deaminated to inosine by an active erythrocyte adenosine deaminase (37), but the reactions involved in the

utilization of 2,6-diaminopurine riboside and xanthosine are not yet known. Since erythrocyte purine nucleoside phosphorylase can apparently also cleave deoxyinosine (33), deoxyribose from the deoxyribosides could also enter a metabolic pathway the details of which have not been elucidated in the erythrocyte. The observation that ribose and deoxyribose were effective in promoting the reduction of methemoglobin to hemoglobin and that lactic acid accumulated when erythrocytes were incubated with these pentoses is of some interest. Other investigators have stated that ribose is not metabolized by erythrocytes *in vitro* (38), although increased oxygen uptake by rabbit erythrocytes incubated with ribose, arabinose and xylose plus methylene blue has been reported (39). The pathways by which ribose and deoxyribose are metabolized are unknown, but they might involve phosphorylation and entry into the hexose-monophosphate shunt and Embden-Meyerhof pathway. Glucose, fructose and galactose are metabolized by these pathways with the regeneration of reduced pyridine nucleotides. Fumaric acid may be metabolized to malic acid by fumarase and the latter can be oxidized to oxaloacetate by malic acid dehydrogenase with the reduction of DPN to DPNH. These two enzymes of the tricarboxylic acid cycle are known to be present in mature mammalian erythrocytes (38). Since the metabolism of these various substrates can lead to the regeneration of DPNH, the reported observations are consistent with the concept that the DPNH-dependent mechanism is the one that normally reduces methemoglobin to hemoglobin in normal human erythrocytes. Differences in the effectiveness of these various compounds may reflect differences in the permeability of the erythrocyte or differences in the rates at which the metabolic reactions proceed within the cell.

When a hemolysate of normal human erythrocytes rather than intact erythrocytes was incubated with some of the effective compounds, only ascorbic acid had an effect on the concentration of methemoglobin after three hours of incubation (Table III). It should be noted that no attempt was made to inhibit the destruction of pyridine nucleotides that is known to occur when animal tissues are disrupted (40). The observation that incubation with ascorbic acid leads to the reduction of methemoglobin in a hemolysate, as well as in intact erythrocytes, is compatible with the belief that ascorbic acid acts directly on methemoglobin (41). This experiment with a hemolysate confirms the importance of an intact erythrocyte for methemoglobin reduction in the absence of methylene blue or other autoxidizable dyes. In addition, it demonstrates that the compounds effective with intact erythrocytes did not exert their effect by reducing methemoglobin to hemoglobin directly.

TABLE III

The effect of various compounds on the reduction of methemoglobin in a hemolysate of normal human erythrocytes *

Compound	Methemoglobin after incubation for 3 hrs.
	%
Control	72
Glucose	74
Glucose-6-phosphate	71
Adenosine	72
Deoxyadenosine	73
Ribose	75
Deoxyribose	70
Ascorbic acid	34

* Initial concentration of methemoglobin was 72 per cent. Concentration of compounds was 19 μMoles per ml. of erythrocytes.

The effect of methylene blue on the reduction of methemoglobin may be due to the ability of this dye to serve in an electron transport system (2). It may, however, also be due, in part, to the activation or stimulation of the hexose-monophosphate shunt pathway (4, 42, 43). The incubation of normal human erythrocytes containing methemoglobin with glucose, inosine or deoxyinosine plus methylene blue resulted in marked acceleration in the reduction of methemoglobin to hemoglobin (Table IV, Part A). The failure of methylene blue to enhance greatly the effect of ribose and deoxyribose is not readily explained. It is conceivable that the permeability of the erythrocyte to ribose is so limited that no acceleration from addition of methylene blue could be demonstrated. If deoxyribose is metabolized by way of the Embden-Meyerhof pathway, only a limited increase in the rate of methemoglobin reduction would be expected since the methemoglobin reduction system that utilizes DPNH is not enhanced markedly by this dye (4, 32, 42).

TABLE IV

The effect of methylene blue on the ability of some compounds to enhance the reduction of methemoglobin in human erythrocytes

Compound	Concentration*	Initial methemoglobin	Methemoglobin after incubation for 3 hrs.	
			Without meth. blue	With meth. blue†
		%	%	%
A. Normal human erythrocytes				
Control		68	64	59
Inosine	10		44	11
Glucose	10		49	3
L (+)-sodium lactate	19		48	34
Control		67	65	62
Ribose	10		58	51
Deoxyribose	10		52	47
Control		65	62	57
Inosine	10		40	9
Deoxyinosine	10		44	14
B. Erythrocytes of congenital methemoglobinemia				
Control		68	77	72
Glucose	10		74	4
Inosine	10		74	10
L (+)-sodium lactate	20		75	69

* Concentration in micromoles per milliliter of erythrocytes.

† Concentration of methylene blue was 0.12 μMoles per ml. of erythrocytes.

This latter effect was also shown by the minimal increase in the effect of L (+)-sodium lactate when methylene blue was added to the erythrocyte suspension.

The specificity of the effect on the reduction of methemoglobin to hemoglobin is demonstrated by those compounds that did not increase the rate of reduction of methemoglobin above that observed in the control suspensions (Table V). Compounds closely related in structure to the effective purine nucleosides were ineffective. Incubation with purine riboside, adenosine-1-N-oxide, adenine glucoside, dimethyl-adenosine, aminonucleoside and Puromycin Hydrochloride®, as well as with the purine, adenine, did not result in either enhanced reduction of methemoglobin to hemoglobin or in the production of lactic acid. A slight effect on methemoglobin reduction was observed with aminoadenosine (Table II) and about 13 per cent as much lactic acid was present after six hours of incubation as with an equimolar amount of adenosine. It remains to be determined if this finding represented true metabolism of the amino-adenosine or if there occurred a small amount of non-enzymatic hydrolysis of the compound with the formation of adenosine. When the purine portion

TABLE V

Compounds ineffective in enhancing the reduction of methemoglobin to hemoglobin when incubated with normal human erythrocytes

Compound	μMoles*	Compound	μMoles*
Pyrimidine ribosides		Intermediates of carbohydrate metabolism and related compounds	
Cytidine	8		
Uridine	8		
Pyrimidine deoxyribosides		Sucrose	9
Deoxycytidine	8		
Deoxyuridine	8	Glucose-6-phosphate	10
Thymidine	8	Fructose-1,6-diphosphate	11
		Ribose-5-phosphate	11
Nucleotides		2,3-diphosphoglycerate	11
Adenosine-5′-phosphate	11		
Adenosine diphosphate	11	Aminoribose	10
Adenosine triphosphate	11		
Adenosine-5′-phosphoramide	10	D (-)-sodium lactate	19
		Sodium pyruvate	17
Related compounds			
Adenine	7	Miscellaneous compounds	
Adenosine-1-N-oxide	8, 10	Aspartic acid	10
Dimethyl-adenosine	10	Cysteine	10
Aminonucleoside	10	Glucuronic acid	10
Adenine glucoside	8, 10	Glucuronolactone	10
Purine riboside	8	Glutathione, reduced	10
Puromycin Hydrochloride®	10	Sodium thiosulfate	10

* Concentration in micromoles per milliliter of erythrocytes.

of the effective compounds was replaced by another group, there was no effect on the reduction of methemoglobin and no lactic acid production was observed (10). The pyrimidine ribosides (cytidine and uridine) and the pyrimidine deoxyribosides (deoxycytidine, deoxyuridine and thymidine) were ineffective. The nucleotides of adenosine (adenosine-5′-phosphate, adenosine diphosphate, adenosine triphosphate and adenosine-5′-phosphoramide) were also without effect on methemoglobin reduction. Incubation with a disaccharide (sucrose), sodium pyruvate, aminoribose and the optical isomer of the natural form of sodium lactate, *i.e.*, D (−)-sodium lactate, did not result in increased reduction of methemoglobin to hemoglobin. Several phosphorylated intermediates of carbohydrate metabolism (glucose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate and 2,3-diphosphoglycerate) were ineffective, probably as a result of the impermeability of the erythrocyte to phosphorylated sugars. Compounds unrelated in structure to the purine nucleosides were studied and were ineffective. These compounds included aspartic acid, sodium thiosulfate, glucuronic acid and glucuronolactone. Although it has been reported that reduced glutathione and cysteine will reduce methemoglobin in hemolysates (44), these compounds were ineffective in the intact erythrocyte system used in these studies.

Erythrocytes of congenital methemoglobinemia

Data from an experiment performed with the erythrocytes from the patient with congenital methemoglobinemia are presented in Figure 2 and Table I, Column B. It is apparent that incubating these erythrocytes with the purine nucleosides and sugars that promoted methemoglobin reduction in normal human erythrocytes did not result in significant reduction of methemoglobin to hemoglobin. Incubation with ascorbic acid, which is thought to act directly on methemoglobin (41), did lead to a decrease in the methemoglobin concentration. The quantity of lactic acid present after six hours of incubation was comparable to that observed with normal erythrocytes, except that perhaps slightly more lactate was produced from glucose. The significance of this apparent increased glucose utilization remains to be investigated. The data on lactic acid production con-

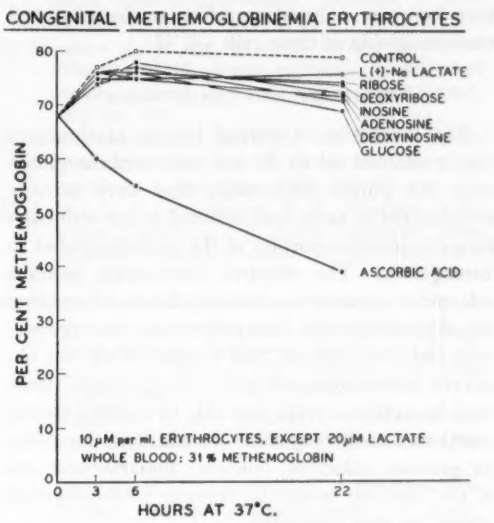

FIG. 2. EFFECT OF INCUBATION WITH VARIOUS COMPOUNDS ON THE REDUCTION OF METHEMOGLOBIN IN THE ERYTHROCYTES OF CONGENITAL METHEMOGLOBINEMIA

firm the observation that the erythrocytes of congenital methemoglobinemia can metabolize glucose (45) and they also demonstrate that these cells can metabolize ribose, deoxyribose and the pentose moiety of certain purine nucleosides.

The nature of the defect in the erythrocytes of congenital methemoglobinemia is unknown, but may involve the absence of an unknown electron carrier system between reduced pyridine nucleotides and methemoglobin (30, 42, 46). That the deficiency can be corrected by methylene blue has been reported by a number of investigators (32, 45, 47) and is further supported by the data in Table IV, Part B. The concentration of methemoglobin in the erythrocytes from the patient with congenital methemoglobinemia was increased by incubating the cells with sodium nitrite. Incubation of these treated erythrocytes with glucose or inosine plus methylene blue resulted in the reduction of methemoglobin to an extent equal to that observed in normal erythrocytes. Only a slight effect from L (+)-sodium lactate and methylene blue was noted. Since the pathways by which nucleosides and sugars are metabolized in the congenital methemoglobinemia erythrocytes would appear to be normal, it may be that methylene blue serves in a substitute electron transport sys-

tem between reduced pyridine nucleotides and methemoglobin in these cells (4, 42).

SUMMARY AND CONCLUSIONS

The incubation of normal human erythrocytes that contained 60 to 80 per cent methemoglobin with the purine nucleosides that were actively metabolized to lactic acid resulted in the reduction of a considerable portion of the methemoglobin to hemoglobin. The effective nucleosides included adenosine, guanosine, inosine, 2,6-diaminopurine riboside, xanthosine, deoxyadenosine, deoxyguanosine and deoxyinosine, and a slight effect was observed with aminoadenosine. The pentoses, ribose and deoxyribose, were also able to enhance the reduction of methemoglobin. In addition, the ability of glucose, galactose, fructose, fumaric acid and L (+)-sodium lactate to promote methemoglobin reduction was confirmed.

The erythrocytes from a patient with congenital methemoglobinemia were unable to reduce methemoglobin to hemoglobin when incubated with the nucleosides and sugars that promoted the reduction of methemoglobin in normal erythrocytes, despite the ability of the congenital methemoglobinemia erythrocytes to produce comparable amounts of lactic acid. These observations are consistent with the concept that the defect in congenital methemoglobinemia lies in a failure in electron transport to methemoglobin.

Incubation with ascorbic acid resulted in the reduction of methemoglobin in normal erythrocytes and in hemolysates, as well as in the erythrocytes of congenital methemoglobinemia.

The addition of methylene blue to normal erythrocyte suspensions containing added glucose or purine nucleosides accelerated the rate of reduction of methemoglobin and permitted the erythrocytes of congenital methemoglobinemia to reduce methemoglobin in a normal manner.

The studies presented here suggest that compounds that can be metabolized by human erythrocytes by pathways that can lead to the reduction of pyridine nucleotides will promote the reduction of methemoglobin to hemoglobin, provided the necessary electron transport mechanism is intact.

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NOREPINEPHRINE INDUCED PULMONARY CONGESTION IN PATIENTS WITH AORTIC VALVE REGURGITATION *

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The regulation of venous blood flow into the heart and the effects of blood volume redistribution upon cardiac function have not been amply defined. While enhanced venomotor tone induced by norepinephrine has been found to increase the volume of a central reservoir in the dog (1), such a volume shift has not been demonstrated in man, except inferentially by the finding of a decrease in the limb venous volume (2).

This type of alteration might be expected to affect cardiopulmonary hemodynamics, particularly in a situation where the volume of this central system has already been expanded. Such a state, exclusive of congestive failure, occurs to a notable degree in aortic regurgitation where deficient valve function fails to limit diastolic volume. Thus, a study of the response to norepinephrine in eight patients with this clinical state has been contrasted with the findings in seven normals.

PATIENT MATERIAL

Eight patients with isolated aortic regurgitation due to rheumatic fever, lacking clinical and hemodynamic evidence of mitral stenosis, were selected for this study. All had substantial left ventricular enlargement with the point of maximum apical impulse occurring in the sixth interspace, at, or within a few centimeters of, the anterior axillary line, while the electrocardiogram showed a left ventricular hypertrophy pattern in each instance. At the time of study, there were no symptoms or signs of heart failure, although three patients had had previous manifestations of decompensation and were subsequently well controlled on digitalis. Seven subjects were chosen for control studies. All had normal hearts by physical examination, X-ray and the electrocardiogram.

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METHODS

All subjects were studied in the fasting state approximately two hours after receiving 0.1 Gm. of pentobarbital sodium. A double lumen catheter was used to measure pressures simultaneously from the pulmonary "capillary" and pulmonary artery. The "capillary" position was assumed to be present when the catheter could not be advanced farther in the lung bed, the tip did not move with the cardiac cycle and a compatible pressure pattern was obtained. A specimen of blood saturated with oxygen was secured from the wedged catheter tip whenever this was possible.

After serial oxygen consumptions showed constancy, 15 mg. of Evans blue dye was injected into the pulmonary artery and collected at two second intervals in a rotating fraction collector from a Cournand needle in the brachial artery. Total collection time was about 50 seconds to accommodate the expected prolongation of the dye curve downslope, and was followed by a simultaneous recording of the systemic arterial, pulmonary arterial and "capillary" pressures with electrically integrated means.

An infusion of norepinephrine, 0.2 μg. per Kg. of body weight per minute, was then begun through a polyethylene tube lying in the subclavian vein and continued for 15 minutes. The electrocardiogram and pressures from the pulmonary "capillary," pulmonary artery and brachial artery were recorded continuously. At 15 minutes, Evans blue dye was again injected. The effect of venous occlusion upon the norepinephrine response was assessed by repeating the infusion in four patients. Sphygmomanometer cuffs had been applied to three extremities at diastolic arterial pressure levels 15 minutes previously and maintained during the subsequent 15 minute infusion.

Since the assessment of the cardiac responsiveness to filling pressure and arterial pressure elevation requires information on the volume of blood which the contracting muscle ejects, the quantity partitioned into the forward and regurgitant flow must be ascertained. While the former is obtained with reasonable accuracy by the Hamilton method, the reliability of current methods for estimating regurgitation in man is not established. The method most thoroughly scrutinized is that devised by Korner and Shillingford (3).

The validity of this method of estimating regurgitant flow depends upon the thesis that the downslope of the descending limb of the indicator dilution curve is fixed for a given cardiac output and central volume. The pres-

ence of valvular incompetence produces a decrease in the gradient of this downslope which is used as a measure of regurgitant flow.

Minor variations of this gradient have been observed between normal subjects with the same forward flow and central volume and place a limit on the accuracy of backflow quantification in a given patient (3). However, when sequential testing is performed, this inaccuracy is diminished (4, 5). While it is generally agreed that small valvular leaks are not reliably detected by this method (6), gross leakage, sufficient to be clinically certain, is usually detectable (5) and thus, the patients chosen were those who appeared to have a substantial, isolated, regurgitant lesion of the aortic valve. Moreover, the modification of this flow in the same model or patient may be fairly accurately detected, if sufficiently large, once one already has an indicator dilution curve for comparative purposes describing the particular system to be modified (4, 5). Since the slopes of the dye curve may allegedly be altered by inadequate flow rates to the fraction collector (7), it is pertinent to note that the volumes collected in the control state were above this minimal rate. As the collection volumes increased after norepinephrine, with and without tourniquets, any change in the dye curve slope would also not be ascribed to this factor. In addition, the qualitative contour of the curve has been found not significantly altered despite varied sampling volumes obtained through simultaneous determinations from the ear, radial and femoral arteries (8).

The calculation of the reciprocal of the slope predicted for a given cardiac output and central volume (Hamilton method) was derived from the Korner-Shillingford regression equation based on 91 indicator dilution curves: $\log 1/s = 0.8766 - 0.0614$ (cardiac output) $+ 1.129 \log$ volume. In the absence of valvular insufficiency or shunts, these authors, based on studies in models and patients, claimed that 1/s is quite predictable. In a modification of the original method (6), the observed slope is calculated from the semilogarithmically plotted curve:

$$1/s = \frac{t_2 - t_1}{\log c_1 - \log c_2},$$

where t_1, c_1, and t_2, c_2 represent the coordinates of two points on the straight line downslope. By setting c_1 and c_2 one logarithmic cycle apart, the denominator is reduced to 1, and the value for the equation is equal to the duration of descent of the downslope to one-tenth of a given point along its course. Total cardiac output is then derived from the product of forward flow and the ratio 1/s (observed)/1/s (predicted). The difference between the total and forward flows is attributed to regurgitant flow.

The most serious objection to this method has been raised by the finding that regurgitation into the narrow rigid proximal chamber of an experimental model may fail to distort the indicator dilution curve significantly from that occurring without valvular incompetence (9). Although such gross inaccuracy has not been reported in man or animal studies, this situation bears consideration in the interpretation of dilution curves. The chamber size and elasticity factors during the infusion of norepinephrine are, in all probability, opposite in direction to those associated with the large error alluded to above for the following reasons. Epinephrine in the dog has been shown with a cinefluorographic technique to increase the left ventricular diastolic area (10), and a similar change may be inferred for norepinephrine from the rise in left ventricular diastolic pressures associated with its use (11). Similar pressure changes occur in man, judged by left atrial mean pressures (12). Since the distensibility of the ventricle is increased by epinephrine (11), such pressure changes would imply a greater diastolic volume in the left ventricle.

The calculation of resistances to forward flow was done by accepted methods, as was the total left ventricular stroke work, with a correction for the contribution of filling pressure to the calculated ventricular work (13). Statistical significance was appraised by the Fischer "t" test method (14).

Since none of the patients selected had mitral stenosis, the use of the pulmonary "capillary" mean pressure as a measure of the filling pressure of the left ventricle appears justified: first of all by its close correspondence to the left atrial mean pressures in man (15); and secondly, by a sufficiently close correlation of this latter parameter with the end-diastolic pressure of the left ventricle to minimize inaccuracy in the interpretation of the relation of filling pressure to stroke work (16). Direct measurement of the force of ventricular contraction, showing a linear relationship with calculated stroke work during the changes induced by norepinephrine, justifies the use of this parameter as a measure of contractility (17).

Calculation of the transaortic valvular pressure difference throughout diastole was not feasible in the absence, in nearly all instances, of a suitable dicrotic notch in the brachial arterial curve to indicate the onset of diastole. Left ventricular pressure curves, not warranted in these patients, would constitute the only means of timing the diastolic phase precisely. Thus, we have employed the end-diastolic systemic arterial pressure in the control period and during norepinephrine infusion, comparing them with their respective pulmonary "capillary" pressures, as an indication of the relative change in the transaortic valvular diastolic pressure difference.

RESULTS

Unusual reactions to the infusion of norepinephrine were not encountered except for a brief episode of sinus arrhythmia in one patient and the transient occurrence of ventricular extrasystoles in another, both occurring early and disappearing some minutes before the injection of Evans blue dye.

As detailed in Tables I and II, a tendency for a decline in cardiac output and increase in central volume was noted in both groups, but in an in-

TABLE I

Hemodynamic response to norepinephrine in seven normal patients

Patient, Age, Race, Sex, B.S.A./M.²	Time	Cardiac output	Central volume	Pulse per min.	Left ventricular stroke work	Mean pressures: Brachial artery	Mean pressures: Pulmonary artery	Mean pressures: Pulmonary "capillary"	Resistances: Peripheral arterial	Resistances: Pulmonary arteriolar
	min.	*L./min.*	*L.*		*Gm. M./beat/M.²*	*mm. Hg*			*dynes-sec.-cm.⁻⁵*	
E. S. 41, NF 1.57	0*	5.39	1.54	70	46.1	80			1,187	
	15	4.67	2.15	60	60.0	100			1,716	
M. S. 38, WF 1.53	0	4.35	1.64	80	47.5	103	13	5	1,893	147
	15	3.78	1.56	71	55.0	125	29	9	2,645	445
N. S. 36, WM 1.88	0	8.69	1.94	85	62.7	90	15	6	827	83
	15	7.45	2.07	72	99.3	146	29	14	1,556	128
J. M. 41, NM 1.65	0	5.67	2.45	71	56.1	90			1,262	
	15	5.69	2.76	64	105.4	155			2,173	
H. N. 34, NF 1.68	0	7.80	1.70	87	55.9	85	15	7	871	82
	15	7.82	1.82	87	93.0	142	26	14	1,455	123
J. G. 25, NM 1.58	0	4.34†		65	44.7	84	11	6	1,554	93
	15	3.19		48	49.2	97	20	11	2,438	226
S. D. 31, WM 1.61	0	4.76	1.66	73	52.2	95	11	5	1,602	102
	15	4.62	1.77	67	94.4	162	15	9	2,802	104
Mean values	0	5.86	1.82	75.9	52.1	89.6	13	6	1,314	101.4
	15	5.32‡	2.02‡	67	79.4	132.4	24	11	2,112	205.2
p				<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	<0.01

* Control period.
† Fick cardiac output.
‡ No significant difference from control period.

consistent manner. The significant bradycardia encountered in normals was not paralleled in aortic insufficiency. That substantial flow leaks occurred in the patients with aortic insufficiency is attested by the finding of a mean regurgitation, 61 per cent of the aortic outflow. In the normal group, no significant leak was exhibited. The infusion of norepinephrine was attended by a significant reduction of the calculated regurgitation, contributing to the finding of a decline in the total flow. Since some leak probably occurs across the mitral valve in most enlarged left ventricles, an anticipated accentuation with norepinephrine would not qualitatively modify the observed findings (12).

In striking contrast to the 5 mm. Hg rise in the pulmonary "capillary" pressure for the normal group, was the rise from 8 mm. Hg to 29 mm. Hg in those with aortic regurgitation. The major part of this change occurred after five minutes of norepinephrine infusion and was usually complete by 10 minutes. As this increment exceeded that of the brachial artery diastolic pressure (mean 4 mm. Hg) wherever the regurgitant flow was diminished, a decline in the aortic-ventricular diastolic pressure difference could account for this flow change. The one patient (A. W.) with the smallest control regurgitant volume exhibited an increase in leak associated with a rise in this pressure difference.

Whereas the small increment in the filling pressure was associated with a substantial rise in the

TABLE II

Hemodynamic response to norepinephrine in eight patients with aortic insufficiency

Patient, Age, Race, Sex, B.S.A./M.²	Time	Aortic outflow	Regurgitant flow	Per cent of aortic outflow	Total flow	Central volume	Pulse	Left ventricular stroke work	Pressures: Brachial artery S	Brachial artery D	Brachial artery M	Pulmonary artery S	Pulmonary artery D	Pulmonary artery M	Pulmonary "capillary" Mean	Trans-aortic diastolic pressure difference change§	Resistances: Peripheral arterial	Resistances: Pulmonary arteriolar
	min.	*L./min.*	*L./min.*	%	*L./min.*	*L.*		*Gm. M./beat/M.²*	*mm. Hg*							*mm. Hg*	*dynes-sec.-cm.⁻⁵*	
D. P. 38, NM 1.82	0	4.03	2.30	56	6.33	2.28	78	53.4	156	63	97	28	13	19	9		1,850	187
	15	3.68	1.64	44	5.32	2.26	78	52.0	174	85	138	57	32	42	36	− 5	3,010	109
C. O. 22, NF 1.64	0	6.91	4.62	66	11.53	3.22	76	108.2	188	38	94	44	22	27	8		1,088	220
	15	6.72	1.14	17	7.86	4.66	82	68.4	250	48	110	65	34	44	24	− 6	1,308	119
R. J. 32, WM 1.70	0	5.80	4.18	72	9.98	4.79‡	82		120	41	60							
	15	4.96	3.47	70	8.43	3.67	78		156	50	92							
J. K. 70, WM 1.68	0	6.03	4.22	70	10.25	2.84	84	68.1	138	55	80	44	12	24	11		1,066	173
	15	4.24	2.14	50	6.38	2.80	79	52.3	175	70	110	63	33	40	30	− 4	2,064	188
H. B. 19, NM 2.07	0	4.83	2.51	51	7.34	2.95	70	53.0	160	50	87	26	11	16	11		1,239	83
	15	5.38	0.38	7	5.76	3.59	73	52.9	268	64	132	48	31	36	30	− 5	1,962	89
	15*	6.58	2.55	37	9.40	3.36	72	86.5	240	62	118	33	16	23	17	+ 6	1,378	70
N. L. 32, WM 1.75	0	5.15	3.96	77	9.11	3.06	63	92.9	111	52	87	17	8	11	4		1,347	108
	15	3.21	1.76	55	4.97	2.60	72	45.6	207	79	116	52	27	38	31	− 1	2,915	176
	15*	4.23	5.27	123	9.50	3.12	70	112.1	207	80	122	34	15	21	16	+16	2,064	113
A. H. 52, NM 2.2	0	3.53	2.86	81	6.49	2.25	83	35.2	135	60	86	61	31	40	12		1,947	633
	15	4.21	2.65	63	6.86	2.72	90	42.4	210	73	118	85	35	55	28	− 3	2,242	480
	15*	4.32	3.15	73	7.47	2.73	83	63.0	240	73	129	61	32	41	16	+ 9	2,390	445
A. W. 53, NF 1.60	0	5.96	1.07	18	7.03	2.63	55	89.0	105	55	86	26	10	16	4		1,160	133
	15	5.20	1.33	25	6.50	2.43	50	124.3	237	80	133	50	20	30	21	+ 9	2,040	139
	15*	2.99	4.00	133	7.00	1.94	50	148.8	260	87	130	21	11	15	5	+31	2,610	266
Mean values	0	5.28	3.09	61.4	8.51	2.75	74	71.4	139	52	85	35	15	22	8		1,385	220
	15	4.70†	1.81	41.4	6.51	3.01†	75†	62.6†	210	69	119	60	30	41	29		2,220	186†
	p		<0.05	<0.02	<0.01						<0.01			<0.01	<0.001		<0.001	
	15*	4.60†	3.74	91.5	8.34†	2.79†	69†	102.5	237	76	125	37	19	25†	13†		2,111	224†
	p							<0.02			<0.01						<0.01	

* With venous occlusion.
† No significant difference from control period.
‡ Not included in mean figures, as dye injection was peripheral rather than central.
§ Change in the difference between brachial artery diastolic and pulmonary "capillary" mean pressures as approximating aortic-ventricular diastolic pressure gradient.

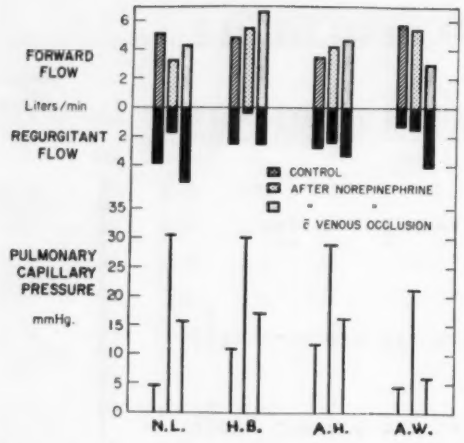

FIG. 1. EFFECTS OF VENOUS OCCLUSION UPON ACTION OF NOREPINEPHRINE

The vertical lines designating pulmonary "capillary" pressure correspond in time to the flow column immediately above.

left ventricular stroke work for the normals, the nearly fourfold pressure rise in the presence of aortic regurgitation involved no higher left ventricular performance level. In an effort to determine whether the response of the pathologic heart could be attributed to volume displacement from the periphery, tourniquets were applied to the extremities of four patients. In these circumstances, as detailed in Table II and Figure 1, the 5 mm. Hg mean rise in the pulmonary "capillary" pressure after norepinephrine corresponded to the finding in normals, as did the substantial increment in the left ventricular stroke work. Thus, hydrostatic pressure levels usually associated with pulmonary edema were effectively prevented by restricting the redistributive ability of the venous system. The increase in the aortic-ventricular diastolic pressure gradient was associated with restoration of regurgitant volumes to control or higher values, confirming the known relationship of transvalvular diastolic pressure difference to regurgitant flow (18).

Although the calculated vascular resistances clearly became elevated in both the total peripheral arterial and pulmonary arteriolar beds in the normal, a change in the latter parameter failed to occur in the valvular insufficiency group after norepinephrine. In a more direct experimental approach, elevation of left atrial pressure has tended to lower resistance in the pulmonary bed (19). Presumably a similar situation has occurred here, with the pulmonary "capillary" reflecting left atrial pressures. As hematocrit changes may affect viscosity and thus alter resistance, it is of note that the hematocrit rose an inconsequential 1 per cent in both groups.

DISCUSSION

The finding of a significant rise in pulmonary "capillary" pressures after norepinephrine in the normal human subject has been ascribed to increases in left atrial pressure (20). Direct evidence supporting this contention has been obtained through left heart catheterization where slight elevations of mean atrial pressures in normals, and substantial rises in pathologic hearts have been observed (21), that are quite comparable to the striking increments of pulmonary "capillary" pressure found in this group with aortic regurgitation. Since pulmonary vein constriction would appear eliminated as a major pathogenetic factor, a direct myocardial depressant effect or displacement of blood from the periphery remain as potential mechanisms. Several sources of evidence would indicate the latter as most probable.

Such a volume shift has been observed in the dog after norepinephrine with an increase in directly measured venous return (15) and in the volume of a reservoir placed distal to the left auricle (1). The cardiopulmonary location of this blood has been further evidenced by increased Hamilton central volumes and left ventricular end-diastolic pressure (11). That a translocation of blood from the periphery is involved in this process would appear to be the case, since limb volumes measured plethysmographically indicate a decline (2, 22).

From the foregoing data one would have expected to find an increase in the calculated central volume. The magnitude of the change observed does not itself confirm a significant central shift of blood. However, the technique of measurement here used does not provide absolute quantification since it fails to account for volumes of blood not displaced by the flow process (23).

A similar disparity has been found in patients with mitral stenosis where no significant increase in calculated pulmonary blood volume occurred

in spite of high pulmonary vascular pressures (24). Moreover, the presence of a methodical error of 15 per cent (25) would indicate that a shift of 400 ml. may escape detection. A rapid increment of this size, in the presence of an already expanded volume, may affect the pressure-volume relationship of the left atrium and pulmonary artery sufficiently to give pulmonary pressures of the order observed, since the shape of the curve describing this relationship is such that the pressure rise exceeds volume increments above an ill-defined limit (26). It may also be noted that others have failed to find a significant volume increment after epinephrine in humans at dosage levels inducing a rise in cardiac output (27), despite the fact that this cardiac effect is largely dependent upon greater venous return (28). Further, the fact that the striking changes in pulmonary "capillary" pressure were prevented by trapping blood volume in the extremities would support the thesis that the pressure rise after norepinephrine was due to volume displacement, despite the failure to find significant expansion of the calculated central volume.

As the directional change of the total output and work of the heart with an incompetent aortic valve showed a decline in the face of elevated filling pressures, these findings correspond to those occurring in low output heart failure (29). The inability of this dilated, hypertrophied myocardium to respond normally to the effects of norepinephrine with an increase in stroke work may be attributable in part, at least, to the dual demands imposed by an increased inflow load and outflow resistance. Interference with the venous return modifies this situation sufficiently to enable a contractility response indistinguishable from that occurring in the normal, presumably by avoiding excessive distention of the ventricle. This further verifies the similarity of this state of norepinephrine induced pulmonary congestion to low output heart failure where an enhanced performance of the myocardium also follows venous occlusion (30). Whether interference with outflow resistance alone would similarly affect the cardiac response is problematic.

In evaluating the response of the cardiac output in this experimental situation, the major source of error resides in the estimation of the regurgitant volume. Since this segment of the total cardiac output would, of necessity, be disproportionately increased for a suitable cardiac response to occur at these filling pressure levels (16, 29), it is difficult to gainsay the conclusion that myocardial performance has been compromised. This represents a relatively severe depression of the ventricular function curve, as it is usually shifted to a higher level by virtue of the direct myocardial activity of most sympathomimetics (31).

While this study illustrates an inappropriate translocation of blood in the presence of a defective, though compensated myocardium, this activity may actually contribute to the effectiveness of norepinephrine in shock states (32). Conversely, the peripheral venoconstriction present in low output heart failure (33) would tend to aggravate pulmonary congestion unless moderated by methods of reducing venous return.

SUMMARY

To assess the pluripotential effects of norepinephrine on the cardiovascular system of man, the response in the normal has been contrasted with that occurring in eight compensated patients with aortic valve regurgitation. Indicator dilution curves were obtained before and during the norepinephrine infusion (0.2 μg. per Kg. per minute) to evaluate the cardiac output, regurgitant flow and central volume. Intravascular pressures from the pulmonary "capillary," pulmonary artery and brachial artery were recorded during the procedure.

A disparity of response between the two groups was most manifest in the pulmonary "capillary" and artery pressure alterations, a nearly fourfold rise to pulmonary congestion levels in the aortic regurgitation group contrasted with a 5 mm. Hg mean rise occurring in the normal. Whereas this small increment of left ventricular filling pressure was associated with a sizable stroke work increase in the normal, no significant change in contractility was found in the aortic regurgitant group, despite markedly elevated filling pressures. Bradycardia and increased pulmonary arteriolar resistance, present in the normals, were also absent in this group.

A consideration of the pathogenetic factors contributing to this situation suggested that displacement of blood from the peripheral venous system

was the predominant, if not sole, mechanism in the induction of pulmonary congestion and diminished left ventricular performance in this circumstance. This interpretation received indirect support from data acquired in four patients during peripheral venous occlusion by tourniquet. Here, the pulmonary "capillary" pressure increment and the stroke work response after norepinephrine were of the same order exhibited in the normal.

A relationship of regurgitation to the aortic-ventricular diastolic pressure difference is inferred from the observation that whenever the pulmonary "capillary" pressure rise exceeded the systemic diastolic arterial response, the calculated regurgitant volume diminished, while this volume was usually increased when the converse pressure relationship existed.

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ALTERATIONS IN METABOLIC ENERGETICS AND CATION TRANSPORT DURING AGING OF RED CELLS

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With the total life span of the human red cell averaging 120 days, about 0.8 per cent of the circulating erythrocytes disintegrate daily. Normal red cell destruction has been attributed to extra- and intracorpuscular factors (1, 2); until recently, little attention has been paid to the intrinsic processes of impaired function in normal red cells that may lead to possible disintegration. The metabolic energy of the red cell is predominantly derived from glycolytic mechanisms and is utilized to maintain transport and membrane functions and to prevent the auto-oxidation of hemoglobin (3). This investigation was designed to ascertain whether alterations in glycolysis and the activity of certain crucial enzymes concerned in the Embden-Meyerhof path occur with the *in vivo* aging of circulating red cells.

Centrifugation of blood aggregates young red cells (including reticulocytes) in the top layers of the packed red cell column (4, 5). Examination of layers of centrifuged red cells after Fe^{59} administration reveals that, five to 15 days later, high radioactivity is incorporated in the top fraction, while at subsequent stages in the life span, the greatest radioactivity is present in the bottom layer (6, 7). Fractionation of red cell samples on the basis of increasing density of the erythrocyte population with age has revealed decreases in content of water, solids and cations (4, 8), lipids (7) and enzyme activity (5) with age. Some chemical and enzyme activities of normal cells are lower than erythrocyte values from subjects during a reticulocyte response (9–12).

Examination of the rate of glucose disappearance, the activity of phosphohexose isomerase, aldolase and lactic apodehydrogenase and the concentration of phosphorylated intermediates of the Embden-Meyerhof path for red cell populations fractionated by differential centrifugation indicates a decrease in glycolytic and enzyme activity for old cell fractions. Energy-dependent cation transport systems also show some diminution.

MATERIALS AND METHODS

Heparinized blood from humans (normal and in response to hemorrhage) was collected in plastic or siliconed glass for centrifugation in blood cell separation tubes at 500 G for 15 minutes; the plasma was removed for respinning to obtain a cell-free preparation and the buffy coat completely removed from the constricted part of the tube. The red cells from the bottom bulb of the tube were suspended in isotonic saline (either buffered or containing dextran or 20 per cent bovine albumin) to make a 40 per cent suspension, and a portion, constituting the original red cells, retained for analysis. After standing for 10 minutes, the remainder of the suspension was spun at 100 G for 10 minutes, followed by 2,000 G for 45 minutes. Where necessary, the estimations detailed have been corrected for the intercellular fluid trapped between the packed cells at different levels in the centrifuged cell column by the Evans blue method (13) in a separate sample spun under identical conditions.

Aliquots of the original cells and the top, middle and bottom 10 per cent fractions of centrifuged cells were measured in calibrated and siliconed pipettes for:

a. Hematologic and chemical investigations. Red cell count (two to four hemocytometer chambers counted), reticulocyte count (per 2,000 red cells) and white cell count (14); hemoglobin-alkaline hemoglobin or cyanmethemoglobin method (15); specific gravity and dry weight of cells; and sodium, potassium, calcium and magnesium estimations by flame photometry (16, 17).

b. Glycolysis and phosphorus compounds. The packed red cells were resuspended in plasma or isotonic buffered phosphate saline with added glucose to give a 40 per cent suspension. The fractions were incubated simultaneously at 37° C. in 25 ml. stoppered flasks in an air phase (a 5 per cent CO_2, 95 per cent O_2 gas phase was used in some experiments) and shaken at 150 cycles per minute through a small amplitude. No hemolysis was found under these conditions. The "true" glucose content of the whole suspension was estimated at zero, one and four hours after incubation at 37° C., and the amount of glucose disappearing and lactic acid formed calculated from the one to four hour period. Glucose was estimated by the method of Somogyi and Nelson (15) or using glucose

oxidase[1] (18) and lactic acid by the method of Barker and Summerson (19); in some cases, pyruvic acid was estimated by the method of Friedemann and Haugen (20) using toluene for specific extraction of pyruvic acid.

Phosphate compounds were estimated on trichloracetic acid filtrates of packed red cell layer fractions for inorganic phosphorus and phosphorus components by differential hydrolysis: seven minutes at 100° C. with N H_2SO_4 ("easily" hydrolyzable phosphorus); 100 minutes at 100° C. with N H_2SO_4 ("difficultly" hydrolyzable phosphorus); and digestion by 60 per cent perchloric acid with heating for 2 hours, followed by addition of concentrated HNO_3 and further heating for 30 minutes (total acid soluble phosphorus). Phosphate was estimated by ascorbic acid reduction of phosphomolybdate. The "easily" hydrolyzable phosphorus corresponds mainly to the two labile phosphorus groups of adenosine triphosphate (ATP); the difference between 100 minute and seven minute phosphorus to hexose, ribose and adenylate phosphorus; and the total acid soluble phosphorus less 100 minute phosphorus to 2:3 diphosphoglycerate (2:3 DPG) predominantly and minor residual phosphate esters. Although differential hydrolysis has not the reliability of chromatographic analysis (21, 22), nevertheless, a preliminary comparison of paper chromatographic and differential hydrolysis methods shows close correspondence for the 2:3 DPG values. While a small amount of hydrolysis of 2:3 DPG in the seven minute or 100 minute estimations would introduce serious error, as commented on (22), we could confirm that this ester was highly resistant to acid hydrolysis (23).

c. Enzyme assays (*Figure 1*). Red cell phosphohexose isomerase was measured from the degree of conversion of 0.002 M glucose-6-phosphate to fructose-6-phosphate in 30 minutes at pH 7.4 and 37° C., following the method of Bodansky (24) and expressed as units per gram hemoglobin. The effect of red cell phosphoglucomutase, phosphofructokinase and phosphatase on the substrate or end-product is negligible. Aldolase was determined from the rate of conversion of fructose 1,6-diphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by the dinitrophenylhydrazine method of Sibley and Lehninger (25). The unit of aldolase activity was expressed in terms of the formation of 1 mg. alkali-labile phosphorus per Gm. hemoglobin per minute, or using glyceraldehyde as a secondary triose standard for correction and calibration, in terms of triose phosphate chromogen (26).

Red cell lactic apodehydrogenase was determined from the formation of diphosphopyridine nucleotide from reduced diphosphopyridine nucleotide (DPNH) during the conversion of pyruvate to lactate. The method of Blanchaer and Baldwin (27) was used and the unit of activity expressed as a decrease in optical density (ΔO.D.) of 1.00 at 340 mμ per Gm. hemoglobin per minute.

[1] High potency enzyme preparations kindly supplied by Ames Co., London, and Dr. L. A. Underkofler, Takamine Laboratories, Clifton, New Jersey.

TABLE I

Physicochemical data for centrifuged red cell layers

	Fraction of red cell column		
	Top 10%	Middle 10%	Bottom 10%
Reticulocyte count* (%)	36	1.2	0
Red cell count (*10⁶/ml. cells*)	9.8	11.9	12.8
Hemoglobin (*Gm./ml. cells*)	0.272	0.320	0.338
Density (*Gm./ml. cells*)	1.102	1.110	1.115
Dry weight (*Gm./ml. cells*)	0.363	0.402	0.420
Water content (%)	67.1	63.8	62.3
Cell weight ($\mu\mu$g.)	112	93	87
Cell water ($\mu\mu$g.)	75	59	54
Cell solids ($\mu\mu$g.)	37	34	33
Cell hemoglobin ($\mu\mu$g.)	27.8	26.8	26.4
Cell volume (*cu.* μ) (from density)	101	84	78
Cell volume (*cu.* μ) (from hematocrit)	108	88	79

* Values based on average of 12 samples with whole population reticulocyte counts of 5 to 10 per cent.

d. Presentation and precision of analyses. Calculation of results in terms of volume (or weight) of red cells, volume (or weight) of cell water or of cell solids does not provide a valid comparison for analyses taken from different layers, especially in the presence of a moderate to marked reticulocytosis. Alterations in the size, water content and solid content of a red cell during its life span are appreciable (4, 28) and the data presented in Table I indicate that results, where possible, should be referred to the hemoglobin content of the particular sample assayed. While synthesized to a slight degree by reticulocytes and undergoing some degradation at the end of the erythrocyte's life span, the hemoglobin content of the red cell remains virtually unchanged. Results expressed per unit number of red cells are subject to the relatively large error for red cell counts.

All readings were made on an Evelyn filter photometer (using the micro attachment when necessary) or the Beckman DU spectrophotometer. The precision of enzyme assays was checked by duplicate estimations; the percentage error ranged from 3 to 10 per cent. Duplicate estimations on red cell samples fractioned for the phosphate partition are presented in Table II. Since values obtained from the differential hydrolysis are interdependent, the analysis was repeated when errors greater than 10 per cent occurred. Errors of 3 to 8 per cent were found for the different phosphate components. For the other chemical determinations, the reproducibility of replicate estimations and the accuracy, assessed by recovery of the substance being determined from additions to red cell suspensions, had errors of less than 2 per cent.

RESULTS

On centrifugation, some 65 to 75 per cent of the total reticulocytes of the original population became concentrated in the top zero to two-tenths fraction of the packed cell column, and 45 to 55

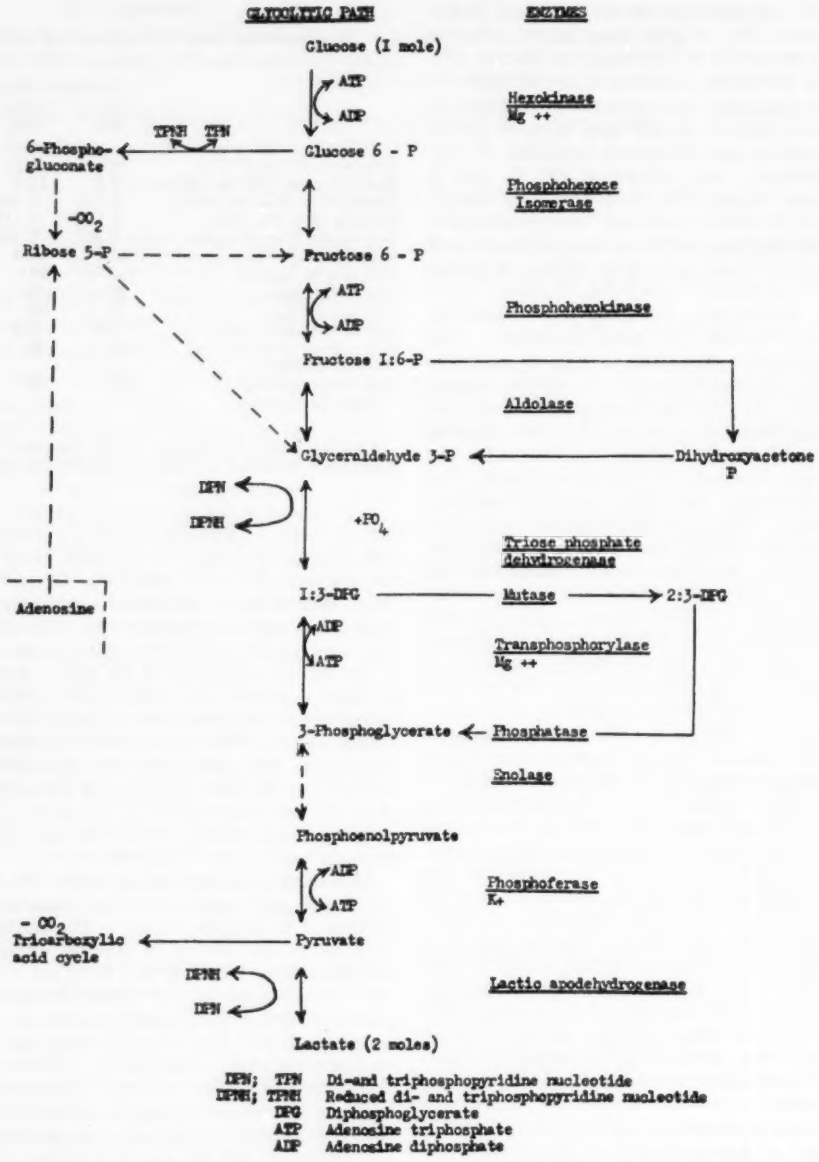

FIG. 1. DEGRADATION OF GLUCOSE TO LACTATE IN THE RED CELL

Broken arrows indicate omission of intermediate steps.

per cent in the top zero to one-tenth fraction. For normal subjects with reticulocyte counts of 0.4 to 1.4 per cent, the top zero to one-tenth fraction contained 2 to 8 per cent reticulocytes. Subjects with a reticulocytosis were mainly cases several days after a hemorrhage, when the level of circulating reticulocytes reached 2 to 10 per cent. Blood samples with a 5 per cent reticulocyte count concentrated a 25 per cent reticulocyte count in the top zone on centrifugation, while specimens

TABLE II

Precision of differential phosphate partition in 50 duplicate red cell samples

Phosphate component*	Sample 6		Sample 11		Sample 18		Standard deviation of difference between duplicates†
	x_1	x_2	x_1	x_2	x_1	x_2	
	μMoles P/Gm. hemoglobin						
Inorg. P	5.15	4.75	4.67	4.90	8.70	8.86	±0.18
EHP (7 min.)	7.15	7.50	6.95	7.25	9.28	8.32	±0.48
DHP (100 min. − 7 min.)	6.06	5.54	4.12	3.87	5.97	5.72	±0.30
2:3 DPG	28.50	29.80	23.24	24.76	22.44	21.56	±1.35

* Inorg. P = inorganic phosphate; EHP = easily hydrolyzable phosphate (mainly adenosine triphosphate); DHP = difficultly hydrolyzable phosphate (hexose and adenylic phosphate); and 2:3 DPG = 2:3 diphosphoglycerate.
† S. D. = $[\Sigma(x_1 - x_2)^2/n - 1]^{\frac{1}{2}}$.

with an original reticulocyte percentage of 10 showed over 50 per cent reticulocytes in the top layer.

The number of reticulocytes decreased sharply in successive fractions and few reticulocytes were found in the five-tenths to one level (Table III).

The possible effect of the presence of white cells and platelets on glycolysis and biochemical values of the various red cell fractions was found to be slight. Bottom layers revealed a virtual absence of platelets and 0 to 6×10^5 leukocytes per ml. cells, *i.e.*, white : red cell ratio of $< 1:20{,}000$; and an occasional platelet and some 8 to 18×10^5 leukocytes per ml. cells for top fractions, *viz.* a white : red cell proportion of about 1 : 10,000. For white : red cell ratios of 1 : 500 to 1,000 in normal whole blood, some 15 to 25 per cent of whole blood glycolysis has been attributed to the leukocytes (29, 30). Contamination of the top fraction of red cells with leukocytes could thus produce a 3 per cent maximum enhancement of red cell glycolysis; errors of a lesser order were calculated for erythrocyte phosphate compounds and cation contents.

Glycolysis

Rates of glucose utilization. The various steps in the red cell glycolytic cycle are depicted in Figure 1. Overall glycolysis was assessed by the amount of glucose degraded and lactate formed. In an initial series of experiments, analyses of packed red cells at fractional intervals showed a

TABLE III

Glucose utilization and lactic acid formation in original and fractionated normal red cells during three hours' incubation at 37° C. in air

	Original cells	Fraction of red cell column					
		0–0.1	0.1–0.2	0.2–0.4	0.4–0.6	0.6–0.8	0.8–1.0
Normals [24]							
Reticulocytes (%)	0.9	6	2.3	0.4	0	0	0
Glucose utilized (*μMoles/Gm. hemoglobin/hr.*)	6.3	11.4	8.8	7.1	6.3	5.2	4.3
Lactic acid formed (*μMoles/Gm. hemoglobin/hr.*)	12.1	19.7	16.7	13.9	11.9		8.3
Conversion (%)	96	86	95	98	94		96
Reticulocytosis [8]							
Reticulocytes (%)	6	28	17	13	0.5	0	0
Glucose utilized (*μMoles/Gm. hemoglobin/hr.*)	7.2	20.0	13.2	9.3	6.6	5.3	4.6
Lactic acid formed (*μMoles/Gm. hemoglobin/hr.*)	13.8	32.7	23.8	17.2	13.0		8.4
Conversion (%)	96	82	90	92	98		91

TABLE IV

The effect of in vitro *incubation at 37° C. in air for 24 hours on the rate of red cell glycolysis*

	Original cells	Fraction of red cell column		
		Top 10%	Middle 10%	Bottom 10%
Beginning of period				
Reticulocytes (%)	2.4	10	0.3	0
Glucose utilized (*μMoles/Gm. hemoglobin/hr.*)	5.9	12.8	6.0	5.1
Lactic acid formed (*μMoles/Gm. hemoglobin/hr.*)	11.2	22.4	11.2	9.4
End of period				
Reticulocytes (%)	0.4	1.6	0	0
Glucose utilized (*μMoles/Gm. hemoglobin/hr.*)	5.2	9.2	5.6	4.5
Lactic acid formed (*μMoles/Gm. hemoglobin/hr.*)	9.7	16.5	10.7	9.1

progressive decrease in glycolytic rate from the top to the bottom layer zone (Table III). For the cases with reticulocytosis, the increased rate of glycolysis in the top fractions was accentuated. The glycolytic rate of the reticulocyte would appear to be approximately eight times that of old cell populations from the bottom centrifuged fraction and five times that of the average whole cell population.

The greater rate of glycolysis in red cells from the top layers was not solely due to the aggregation of reticulocytes in these fractions, since the glycolytic rate was higher in the four to six-tenths and six to eight-tenths fractions compared with the eight-tenths to one fraction layers where the reticulocyte was extremely scanty. The aging of red cells with progressive decrement in glycolytic activity was further indicated by the following experiment. Red cells from the original suspension and the top, middle and bottom 10 per cent fractional parts of blood samples with a reticulocytosis were incubated for 24 hours at 37° C. with estimation of the glycolytic rate at the beginning and end of the incubation period. Probably due to *in vitro* deterioration, there was a relative decrease in glycolytic rates of all fractions at the termination of incubation (Table IV). However, the high glycolytic rate of the top fraction after 24 hours' incubation was noteworthy, since the reticulocytes had virtually disappeared from all fractions owing to maturation. Seip (31) concludes that two-thirds to three-fourths of circulating reticulocytes lose their reticulum in 30 hours. The supposition is that the decrease in glycolytic activity progressed with aging of the red cells.

The production of lactate was approximately equivalent to the amount of glucose degraded in bottom fraction cells. The lower percentage conversion of glucose to lactate in the top fraction of the cell column suggested divergence of glycolytic intermediates into other metabolic paths in young cells (see Discussion). The final pH of the suspension was 0.1 to 0.3 pH units lower at the end of incubation, depending on the degree of glycolytic activity, and was higher for the air than the 5 per cent CO_2, 95 per cent O_2 gas phase. Longer periods of incubation, despite the provision of additional buffer in the suspension systems, produced decreases of 0.5 to 0.8 pH units with some inhibition of glycolysis.

In a further series of experiments, top and bottom fraction cells were incubated in various media (Table V). Suspension of cell fractions, in solutions containing fructose, mannose or galactose instead of glucose and incubated at 37° C., resulted in entry of these sugars into red cells as judged by their decreasing concentration in the external medium and their metabolism as evidenced by acid production. Sugar degradation was greater in the top fraction independent of the suspending medium and greater lactic acid formation occurred in the suspension from the top portion. Fructolysis and formation of acid was marked

TABLE V

Rates of sugar degradation for top and bottom red cell fractions from normal blood sample suspended in various media

Suspending medium	Top fraction		Bottom fraction	
	Sugar utilized	Lactic acid formed	Sugar utilized	Lactic acid formed
	μMoles/Gm. hemoglobin/hr.		*μMoles/Gm. hemoglobin/hr.*	
Glucose-phosphate-saline*	11.6	20.1	5.2	10.0
Fructose-phosphate-saline†	12.1	20.2	4.8	9.1
Mannose-phosphate-saline‡	8.0	14.8	3.6	6.3
Galactose-phosphate-saline‡	2.2	5.4§	0.9	3.0§
Adenosine-phosphate-saline‖		18.6		8.5
Plasma, heparinized	10.9	19.2	4.8	8.9
Plasma, oxalated	8.6	16.5	4.0	7.8

* Concentration of hexose sugars, 2 mg./ml.
† Estimation by Seliwanoff resorcinol-HCl method (Reference 15, p. 229).
‡ Estimation by glucose method after fermentation, using appropriate factor (15).
§ Includes lactic acid formed from cell hexose phosphate.
‖ Concentration of adenosine, 1 mg./ml.

and similar to glucose in degree. The utilization of mannose was less and the metabolism of galactose was of slight degree. Adenosine or inosine could replace glucose either wholly or in part as a substrate for glycolysis. Purine nucleosides are metabolized by red cells, forming ribose-5-phosphate which may enter the glycolytic pathway (Figure 1). Both oxalate and ethylenediamine tetraacetate were considered unsuitable as anticoagulants since they depressed glycolytic rates considerably.

Phosphorylated compounds and glycolytic enzymes. The phosphate partition of the various centrifugated fractions is presented in Table VI for specimens of blood from 18 normal subjects and seven cases with reticulocytosis. The high concentration of 2:3 DPG is peculiar to certain mammalian red cells and represented some 60 per cent of the total phosphate analyzed, with a further 15 per cent for the readily hydrolyzable phosphate, corresponding mainly to the two labile groups of ATP. The 2:3 DPG represents a stable store of phosphate ester (Figure 1) with a high rate of turnover (22, 23). The top fraction of centrifuged cells had a higher concentration of 2:3 DPG than lower levels in 16 of the 18 normal samples, exceeding the 95 per cent confidence limits for the reproducibility of duplicate estimations given in Table II. The difference of the means, 4.0, was significant, since it exceeded three times its standard error, ± 0.86. The readily hydrolyzable phosphate also tended to have higher concentrations in the top strata of the centrifuged cell column, 13 values exceeding 95 per cent confidence limits for the reproducibility of estimations. The difference of the means, 2.1, was significant by comparison with its standard error, ± 0.21. These deviations were accentuated in the cases of reticulocytosis examined. For other phosphate components (inorganic, difficultly hydrolyzable and total acid soluble phosphate), no clear-cut difference was evident for the various fractions.

When aliquots from different strata of packed cells were washed in isotonic glucose-free solutions and incubated without glucose, the result was a rapid decrease in 2:3 DPG content with a slower diminution in ATP, and a concomitant

TABLE VI

Phosphate partition in red cell fractions

Phosphate component*	Fraction of cell column			
	Normals [18]†		Reticulocytosis [7]‡	
	Top 10%	Bottom 10%	Top 10%	Bottom 10%
	μMoles P/Gm. hemoglobin			
Inorg. P	4.7 ±0.42	5.6 ±0.30	4.1 (3.0–5.5)	5.2 (4.4–6.3)
EHP	9.1 ±0.68	7.0 ±0.55	11.8 (8.1–14.6)	7.3 (6.2–8.0)
DHP	4.5 ±0.32	4.7 ±0.36	5.1 (4.3–6.1)	4.1 (2.9–5.0)
2:3 DPG	29.9 ±2.4	25.9 ±2.7	33.7 (29.2–38.1)	23.8 (19.2–29.5)

* See footnote, Table II.
† Values are means of duplicate estimations ± S. D.
‡ Mean and range of duplicate estimations.

increase in the inorganic phosphate content of the cell. Table VII depicts the changes that occur in the initial three hour period of incubation for one of five experiments with similar results. Approximately 10 per cent and 20 per cent of 2:3 DPG were catabolized hourly in bottom and top fraction cells, respectively. Since the conversion of 2:3 DPG to pyruvate involves the formation of 1 Mole ATP (easily hydrolyzable phosphate) for each Mole 2:3 DPG dissimulated (Figure 1), the total loss of ATP from the system was represented by the combined ATP and 2:3 DPG decrease. For top fraction cells, the loss was nearly twice that for the bottom fraction, indicative of the greater inherent metabolic activity of upper layer erythrocytes. The difference between the mean decrease in five experiments of 2:3 DPG (and total ATP loss) was highly significant, exceeding six times the standard error of the difference.

The degradation of the phosphate esters and the formation of lactic acid decreased progressively with continued incubation, the hourly rate of change for six hours' incubation being about 60 per cent of that calculated for the three hour period. With longer periods of incubation, increasing rate of disappearance of easily hydrolyzable phosphate with rise in inorganic phosphate heralded the onset of hemolysis.

Apart from small amounts of hexose and pentose phosphates in the red cell, the 2:3 DPG provided the major source of energy (equivalent to 50 to 70 per cent of the lactic acid formed) in the absence of glucose or other metabolizable sugar in the external medium. Some accumulation of pyruvate occurred in cells derived from the bottom layer of the centrifuged column (Table VII). Since pyruvate accumulation was not found in the same red cell fractions in the presence of glucose, it was postulated that the decrease of conversion of glyceraldehyde 3-phosphate to 2:3 DPG in glucose-free media created a diminution in the formation of DPNH and a relative deficiency of DPNH (coenzyme) for lactic apodehydrogenase conversion of pyruvate to lactate (Figure 1). Analogous mechanisms to explain pyruvate accumulation have been suggested by Whittam (32) for fresh red cells incubated without glucose and by Blanchaer and Baldwin (27) for slowly glycolyzing cold-stored cells. Pyruvate accumulation was not as evident in the upper fraction of red cells incubated in glucose-free media, presumably since pyruvate in reticulocytes can be metabolized by other routes.

TABLE VII

Incubation of normal red cell fractions at 37° C. for three hours without glucose

Phosphate and glycolytic components*	Top fraction	Bottom fraction
Initial 2:3 DPG (*μMoles P/Gm. Hb*)	30.2	22.5
Δ 2:3 DPG (*μMoles P/Gm. Hb/hr.*)	−4.3	−2.3
Initial EHP (*μMoles P/Gm. Hb*)	8.5	6.8
Δ EHP (*μMoles P/Gm. Hb/hr.*)	−0.5	−0.3
Δ Inorg. P† (*μMoles P/Gm. Hb/hr.*)	+4.5	+2.8
Δ Pyruvate (*μMoles/Gm. Hb/hr.*)	+0.2	+0.6
Δ Lactic acid (*μMoles/Gm. Hb/hr.*)	+7.2	+3.5

* See footnote, Table II; Hb = hemoglobin.

† This is estimated from the observed change in cell inorganic phosphorus plus cell phosphorus lost by diffusion, evaluated by difference in total cell phosphorus before and after incubation.

Spinning the cell aliquots and resuspension in solutions containing adenosine (or inosine) for incubation at 37° C. led to a regeneration of the ATP (easily hydrolyzable phosphate) and 2:3 DPG; the former reached nearly original values in 30 minutes and the latter by 120 minutes, with the upper fraction cells having higher concentrations than bottom cells. Resuspension in glucose-inorganic phosphate buffer resulted in a slower and less complete resynthesis of these phosphate esters.

The enzyme activities of phosphohexose isomerase and aldolase showed a progressive decrease from young (top fraction) to old (bottom fraction) red cell populations. Values for different studies are collected in Table VIII and indicate that the top fraction of cells from normal blood had an enzyme activity some 80 per cent greater than bottom fraction cells. On this basis, the reticulocyte phosphohexose isomerase and aldolase activity was several times that of the general cell population. Fractional parts, incubated at 37° C. for 24 hours and reanalyzed, showed some 20 per cent decrease in enzyme activity. However, while few reticulocytes could be detected in the top fraction (having undergone maturation), the enzyme activity of this portion was still greater than that for the other layers, indicating the progressive nature of the loss of activity for these enzymes with aging of the red cells.

Lactic apodehydrogenase activity, on the other

TABLE VIII

Enzyme assays in red cell fractions

	Reticulocytes	Phosphohexose isomerase*	Aldolase†	Lactic apodehydrogenase*
	%	*10³units/Gm. Hb*	*units/Gm. Hb/min.*	*Δ O.D./Gm. Hb/min.*
Normals, No. of values	17	14	8	17
Original suspension	0.8	12.8 ± 1.5	0.29	158 ± 22
Top 10% fraction	4.2	19.4 ± 2.6	0.48	180 ± 31
Middle 10% fraction	0.2	14.5 ± 2.2	0.30	150 ± 26
Bottom 10% fraction	0	11.6 ± 1.8	0.23	169 ± 18
Subject M. A.				
Original suspension	3.5	10.6	0.18	192
Top 10% fraction	15.5	25.5	0.38	198
Bottom 10% fraction	0	8.2	0.14	174
Subject W. F.				
Original suspension	9.5	14.6	0.24	143
Top 10% fraction	42.0	35.3	0.53	135
Bottom 10% fraction	0	11.9		152
Original suspension‡	1.8	12.9	0.17	165
Top 10% fraction‡	7.2	20.5	0.32	150
Bottom 10% fraction‡	0	10.7		176

* Mean ± S. D.
† Mean.
‡ Estimations 18 days later.

hand, showed little difference between the various fractional parts from the same blood sample.

In five cases of reticulocytosis examined during the episode and subsequently, the glycolytic rates and enzyme activity (phosphohexose isomerase and aldolase) of the isolated top fraction of cells had decreased considerably from the values during the reticulocytosis, as had the degree of reticulocytosis (Table VIII).

Cation transport

The red cell potassium and sodium values for the different cell fractions are presented in Table IX. The top fraction (reticulocytes and young mature erythrocytes) had a higher potassium and lower sodium than cells from the bottom fraction. The concentrations so obtained represented the cation steady state of the fraction of red cells analyzed, so that the transport of cations into and out of red cells was decreased in the more mature cells. The potassium and sodium contents, considered however in terms of concentrations in cell water, indicated that there was no change in osmotic relations.

The calcium content of red cells was small and no variation in values could be detected among

TABLE IX

Cation contents of top and bottom fractions from normal centrifuged cells

	Mean values in red cells	
	Top fraction	Bottom fraction
Potassium (*mMoles/L. red cells*)	112	97
Sodium (*mMoles/L. red cells*)	11	20
Potassium (*mMoles/L. cell water*)	161	149
Sodium (*mMoles/L. cell water*)	16	31
(K + Na) (*mMoles/L. cell water*)	177	180
Potassium (*μMoles/Gm. hemoglobin*)	415 ± 22 (28)*	294 ± 15 (28)
Sodium (*μMoles/Gm. hemoglobin*)	39 ± 5 (28)	64 ± 8 (25)
Calcium (*μMoles/Gm. hemoglobin*)	3.6 ± 0.5 (18)	3.2 ± 0.8 (18)
Magnesium (*μMoles/Gm. hemoglobin*)	12.4 ± 2.2 (11)	7.8 ± 1.6 (9)

* Standard deviation with number of estimations in parentheses.

TABLE X

Effect of refrigeration on phosphate components and cation contents of red cell fractions in ACD medium

Day of refrigeration	Glycolysis		Phosphate components*						Cation			
			EHP		2:3 DPG		Inorg. P‡		K		Na	
	T†	B	T	B	T	B	T	B	T	B	T	B
	μMoles/Gm. Hb/hr.		*μMoles P/Gm. hemoglobin*						*μEq./Gm. hemoglobin*			
1	0.8	0.2	7.3	6.3	28.6	24.7	5.0	4.2	400§	320	33	52
3			6.7	5.0	25.8	21.4	7.8	6.7	318	246	102	108
5	0.6	0.3	6.2		24.0	18.9	9.6	9.5	264	188	156	187
10	0.4	0.2	6.0	4.4	21.8	15.3	11.3	13.4	186‖	93	232	271

* See footnote, Table II.

† T = top 10% fraction; B = bottom 10% fraction; Hb = hemoglobin.

‡ This is estimated from the observed change in cell inorganic phosphorus plus cell phosphorus lost by diffusion, evaluated by difference in total cell phosphorus before and after incubation.

§ Initial concentrations were: K, 116 and 104; Na, 9.5 and 17 mEq./L., top and bottom cells.

‖ Final concentrations were: K, 54 and 30; Na, 67 and 88 mEq./L., top and bottom cells.

the different fractions. In the case of red cell magnesium, the series analyzed to date is small, but the results suggested some decrease of this element in the old cell populations.

Fractions from the different layers were suspended in preservative media [acid citrate dextrose (ACD); ACD with adenosine or inosine; and the "synthetic" plasma medium of Raker, Taylor, Weller and Hastings (33)] and refrigerated at 5° C. for various times. Potassium and sodium diffused across the red cell membrane according to their electrochemical gradients so that sodium accumulated and potassium decreased intracellularly (Table X). Despite the larger cation concentration gradient of top fraction cells, the bottom cell layer lost more potassium and gained more sodium. This could be attributed to active cation transport being slightly greater in the top fraction cells. While red cell glycolysis at 5° C. proceeded at a low rate, *viz.*, 0.2 to 1.0 μMole glucose dissimulated per Gm. hemoglobin per hour, less than 10 per cent of the rate at 37° C., the greater glycolysis of top fraction cells and the lesser disappearance of easily hydrolyzable phosphate (ATP) and 2:3 DPG during refrigeration linked the energy of glycolysis to active cation transport. Iodoacetate and fluoride abolished these differential effects in refrigerated top and bottom cell fractions on glycolysis, phosphorus esters and cation concentrations, while cardiac glycosides (which affect carrier-transport systems) eliminated the differential cation effects only (to be published).

Samples removed daily from the refrigerated specimens provided a series of top and bottom cell fractions with progressive alteration of potassium and sodium content from their original values depending on the time of refrigeration. The gross accumulation of potassium and extrusion of sodium after incubation of a representative refrigerated sample for 6 hours at 37° C. is given in Table XI. Since the initial cation concentrations of top and bottom fractions before storage were not too widely divergent, the dependence of active and passive transport on the cellular and external concentration of these ions can be assumed to be similar. Passive transport at 37° C. has been assessed in the same cell fractions by two methods: *1*) incubation of an aliquot with cardiac glycosides to inhibit transport systems (34); and *2*) suspension of high potassium (sodium deficient) and high sodium (potassium deficient) cells in isotonic choline chloride under the same conditions. Results of a similar order were obtained. The gross gain or loss of a particular cation plus the passive transport was considered a reasonable estimate of active transport.

As the period of storage was prolonged, so the ability of the red cell to metabolize glucose deteriorated on incubation. This pointed to a progressive defect in intracorpuscular metabolism, which adenosine could repair more effectively than glucose alone (Table XI). The glucose utilization of the bottom cell fraction was invariably less than samples from the top fraction; allied to this was the finding that the top fraction of cells accumulated more 2:3 DPG than those from the bottom part during incubation.

Active transport of cations proceeded at a greater rate in the top fraction cells from each time level of storage. Rates of active transport decreased as the cells regained their normal potassium content and extruded sodium; hence the rate of accumulation of red cell potassium and extrusion of sodium decreased with longer times of incubation. Sodium extrusion was always greater than the potassium influx, with restoration of the original cell volume. Active transport rates on incubation were lower for erythrocytes cold-stored longer than 10 days.

DISCUSSION

Centrifugation or sedimentation of red cells provides a density gradient whereby discrete populations of erythrocytes with different mean ages can be obtained from blood samples. The fractions are not cytochemical or physiological homogeneous populations (28), but they possess a relative concentration of young cells in the upper layers and old cells in the bottom levels. A further method of fractionating red cells into separate age populations is by osmotic lysis (4, 35–37), since young cells have been shown to be less fragile osmotically than old cells. Both methods have been validated by Fe^{59} tagging of newly formed cells of a limited age span (37). While the osmotic fragility method is reported to be more effective in providing fractions with greater enzyme differences for mean cell ages (5), separation by this technique is only suited to experiments not requiring intact cells. The method is therefore inapplicable to studies on metabolic processes in intact red cells.

The mature red cell loses metabolic properties possessed by reticulocytes, *viz.*, ability to incorporate glycine (38) and protoporphyrin (39) into heme; respiration due to mitochondria (4) and the enzymes of the tricarboxylic acid cycle (40); and the presence of ribosenucleic acid with the synthesis of stromal proteins (41, 42). Hence the mature erythrocyte would appear to lack the capability of oxidative processes and synthesis of enzymes after the reticulocyte stage.

The metabolic energy of erythrocytes is derived solely from the utilization of sugar, whether glucose, fructose, mannose or ribose from adenosine and other purine ribosides. Glucose utilization by normal red cells from the bottom strata of centrifuged samples was some 4.3 μMoles per Gm. hemoglobin per hour (corresponding to 1.4 mMoles per L. red cells per hour); reticulocytes could degrade eight times more glucose. Since no alteration occurs in the availability of glucose as substrate, the source of deterioration must be sought in the process of erythrocytic glucose metabolism.

TABLE XI

Potassium accumulation and sodium extrusion of refrigerated, fractioned red cell suspensions incubated at 37° C. for six hours

Sample*		Glucose utilization	Cation transport†					
			Potassium gain			Sodium loss		
			Gross	Passive	Active	Gross	Passive	Active
		μMoles/Gm. Hb/hr.	*μEq./Gm. Hb/hr.*			*μEq./Gm. Hb/hr.*		
A	Top	10.8	6.1	6.5	12.6	7.6	8.3	15.9
	Bottom	5.1	2.9	6.1	9.0	5.2	6.4	11.6
B	Top	10.1	9.6	5.9	15.5	12.2	6.6	18.8
	Bottom	5.3	4.0	4.9	13.3	8.7	6.1	14.8
C	Top	9.3	9.9	4.6	14.5	11.8	5.9	17.7
	Bottom	4.0	4.4	4.9	9.3	5.2	5.8	11.0
D	Top	8.6	7.0	3.3	10.3	9.6	5.3	14.9
	Bottom	3.4	5.1	2.5	7.6	3.8	5.2	9.0
D‡	Top	11.3	12.5	4.0	16.5	15.8	6.0	21.8
	Bottom	5.5	8.4	3.5	11.9	12.5	4.4	16.9

* A, B, C and D represent samples stored for three, five, seven and 10 days, respectively, and having cation compositions of similar order to those given in Table X. Incubated in medium containing: Na, 150 mEq. per L.; K, 5 mEq. per L.; and glucose, 2 mg. per ml. in buffered phosphate at pH 7.4.

† Transport rates may be converted to μEq. per ml. red cells per hour by using an approximate factor of 0.29 and 0.33 Gm. hemoglobin (Hb) per ml. cells for top and bottom fractions, respectively.

‡ D contained in addition, adenosine, 1 mg. per ml.

The conversion of glucose to lactate was near the theoretical equivalent in bottom fraction cells, indicating that the degradation of glucose in the aged cell followed the Embden-Meyerhof route of anaerobic glycolysis. The lesser degree of conversion to lactate for top fraction cells may be attributed to two processes: *a*) Glucose metabolism is partly deviated via the oxidative pentose phosphate path (43) before re-entering the anaerobic glycolytic sequence (Figure 1). By this route, 3 Moles glucose will be degraded to 5 instead of 6 Moles pyruvate formed via the complete Embden-Meyerhof path. Using glucose-1-C^{14} and glucose-6-C^{14}, the proportion of glucose that is catabolized directly to lactate-C^{14} and the fraction that is first shunted via the oxidative pentose phosphate route ($C^{14}O_2$) before re-entering the glycolytic path can be ascertained. Murphy (44) calculated that 10 to 20 per cent of glucose metabolized by human red cells followed the oxidative pathway. Since the glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activity of the most resistant (youngest) to the least resistant (oldest) cells to osmotic lysis ranged from 230 to 20 per cent and 165 to 80 per cent of the whole cell population, respectively (5), and methylene-blue treated top layer cells produced one-third more O_2 than bottom fraction cells (45), the reticulocytes and young mature cells from the top layer have a more active metabolism through the shunt than aged cells.

b) Pyruvate is partly deviated via the Krebs tricarboxylic acid cycle with liberation of CO_2. This mechanism is confined to the reticulocytes since only these circulating red cells contain enzymes of the cycle (3) and mitochondrial-like structures (4). In particular, the respiration of the reticulocytes is some 30 to 50 times that of the mature red cell, in which it is negligible (1, 42).

The different rates of glycolysis in fractional levels of the red cell column, together with glycolytic rates during the maturation of reticulocytes (Table IV), are suggestive evidence of the progressive biochemical maturity of circulating red cells from reticulocytes through young mature to the aged erythrocyte.

The concentration of the two high energy phosphate esters, *viz.*, 2:3 DPG and ATP, in the red cell decreased in the older age fractions of red cells. This is in agreement with the work of Hofmann and Rapoport (46) who found that rabbit reticulocytes had two to three times the ATP content of the average circulating erythrocyte. The human red cell contains a considerable store of 2:3 DPG with a high energy content (ΔF, 16,000 calories). Through conversion to pyruvate, 2:3 DPG is available to generate the formation of ATP required to prime the hexokinase reaction in the initial phosphorylation of glucose. Without a high level of ATP present for this reaction, the subsequent steps leading to the net synthesis of ATP in the overall glycolytic process cannot proceed. The inherently greater metabolic activity of top fraction cells was evidenced by the greater disappearance of these phosphate esters during incubation without glucose. Subsequent addition of glucose and inorganic phosphate or adenosine (or inosine) led to a more rapid resynthesis of phosphate esters and restoration of glycolytic activity in top fraction cells.

Control of the amounts of glucose degraded must depend ultimately on the levels of enzyme activity in the reactions concerned. Since no alteration in lactic apodehydrogenase activity was found, confirming the results of Marks, Johnson and Hirschberg (5) and Rubinstein, Ottolenghi and Denstedt (40), and the amounts of red cell pyruvate were small (0.5 μMole per Gm. hemoglobin), no defect in the conversion of pyruvate to lactate was found. While the concentration of phosphohexose isomerase and aldolase decreased from the top to the bottom strata of red cells, consideration must be given to the possibility that the concentrations of these enzymes were in such excess that they were not rate-limiting. Marks, Johnson and Hirschberg (5) and Löhr and co-workers (47) reported decreases of these and other red cell enzymes with age while this work was in progress. For overall glycolysis, cells from the bottom strata of centrifuged samples produced some 8 to 9 μMoles lactate per Gm. hemoglobin per hour (*i.e.*, 3 mMoles per L. red cells per hour). In the enzyme assays, the substrates were added in excess, and calculation of the amount of triose equivalents transformed *under these circumstances* may be made. Lactic apodehydrogenase activity was computed in terms

of pyruvate formed by converting the alteration in optical density (O.D.) of reduced diphosphopyridine nucleotide (DPNH), using 6.22 for the molar extinction coefficient. For the normal values given in Table VIII, some 1,450 to 1,750 μMoles DPNH per Gm. hemoglobin per hour was converted, *i.e.*, 1,450 to 1,750 μMoles lactate formed per Gm. hemoglobin per hour. For phosphohexose isomerase activity (unit = conversion to 25 μg. fructose-6-phosphate in 30 minutes), 7,100, 6,440 and 10,800 μMoles triose equivalents per Gm. hemoglobin per hour could be transformed for original, bottom and top fraction cells. In the case of aldolase activity, triose equivalents formed were 560, 440, 580 and 930 μMoles triose per Gm. hemoglobin per hour for original, bottom, middle and top fractions, respectively. Recalculation of the results given by Sibley and Lehninger (25) for rat red cell aldolase activity yields a value of 240 μMoles triose formed per Gm. hemoglobin per hour for their conditions. For other enzymes concerned in the Embden-Meyerhof path, Solvonuk and Collier (48) reported pyruvic phosphoferase activity for human red cells to be 130 μMoles pyruvate liberated per ml. cells per 10 minutes, or approximately 2,400 μMoles pyruvate produced per Gm. hemoglobin per hour.

The limitation to overall glycolysis must thus arise from rate-limiting enzyme activity in the sequence of glycolysis. Of the enzymes tested, only aldolase activity was low (1 per cent of muscle aldolase activity and about 10 per cent of the activity present in most tissues) and reduction of aldolase activity in old cells may reach a critical level to limit overall glycolysis. Aldolase activity would qualify as one of the rate-limiting steps since phosphohexose isomerase was present in amounts considerably larger than necessitated by its requirement in glycolysis, while the pentose phosphate path is largely confined to reticulocytes. Calculation of values from (5) lead to estimates of 300 and 600 μMoles triose formed per Gm. hemoglobin per hour for normal and reticulocytosis specimens by this route.

However, the possibility that low hexokinase activity may be present in red cells is being currently investigated for various age fractions of cells and cases with reticulocytosis. Calculated from Crane and Sols' report (49), the red cell hexokinase activity would indicate the formation of 24 μMoles triose per Gm. hemoglobin per hour. Thus the preliminary step in phosphorylation may be the rate-limiting step for overall glycolysis. The requirement of ATP to prime the initial stage of glycolysis may also play a part in the limitation of glycolysis. High concentrations of ATP are required in the hexokinase reaction and these were found to be diminished in the bottom (old) fraction of centrifuged cells.

The supply of a metabolizable sugar is essential to the energy requirement of active cation transport (3, 50–52). Metabolic inhibitors acting at various stages of the pathway of glucose catabolism inhibit active cation transport in varying degree (50). The top fraction (young) human red cells maintained a steady state with higher cell potassium and lower sodium than bottom fraction cells; this may relate to the greater energy supply of the reticulocytes and young mature erythrocytes in the former component.

During refrigeration the greater glycolysis and cation transport of top fraction cells overrode the higher diffusion gradients of potassium or sodium for these cells, and thus the decrease of red cell potassium of top fraction cells was less than for lower strata cells. Incubation of refrigerated cells led to a more rapid rate of glycolysis and more active accumulation of potassium and extrusion of sodium for top fraction cells. While the turnover of potassium and sodium in the red cell is best studied with isotopic tracers, these were not available at the time of the experiments. The data presented were for gross movements under conditions of red cell cation content not met with *in vivo* and, as such, gave only limited information.

The energy of glycolysis, stored as ATP, is coupled to reactions that maintain active cation transport and the integrity of membrane structure necessary for cell survival. Anaerobic glycolysis yields 2 Moles ATP, with a free energy value in tissues of 22,000 to 24,000 calories, for every Mole glucose metabolized (53). With complete conversion of glucose at the rate of 4.3 μMoles per Gm. hemoglobin per hour to lactate in bottom (old) fraction cells, some 0.095 calories per Gm. hemoglobin per hour (29 calories per L. red cells per hour) were available. Since reticulo-

cyte glycolysis was some eight times greater and a proportion followed the oxidative pentose phosphate and tricarboxylic acid routes, the actual energy yield for reticulocytes was considerably greater. The decrease in glycolytic activity with aging of the red cell may lead to a critical level at which the integrity of membrane function can no longer be maintained. Since a red cell lacking active transport would result in a cell with potassium and sodium ratios approaching $[Cl]_{PI}/[Cl]_{RBC} = 1.44$ (54), *i.e.*, $[K]_{RBC}/[K]_{PI}$ and $[Na]_{RBC}/[Na]_{PI}$ of this order with swelling of the cell, deterioration of active cation transport fluxes would lead to alterations in membrane permeability. Decrease of phosphate esters produced an increase in cell inorganic phosphate and diffusion of the inorganic phosphate extracellularly; the decrease in organic cell phosphate would decrease the internal colloid osmotic pressure and decrease the cell volume. Decrease in glycolytic activity may thus diminish the ability of the red cell to stabilize its volume against variations in membrane permeability.

The level of glycolytic activity may play an important role in the oxygen carrying ability of hemoglobin. In hemolysates, hemoglobin is rapidly auto-oxidized to ferric nature, but in the intact cell the reduced pyridine nucleotides (DPNH and TPNH) act as cofactors to the flavoprotein methemoglobin reductase to maintain hemoglobin in the reduced state (3). With decreased glycolysis in aged cells, the generation of reduced di- and triphosphopyridine nucleotides may diminish to subthreshold levels for the cyclic reduction of methemoglobin (Fe^{+++}, non-oxygen carrier) to hemoglobin (Fe^{++}, oxygen carrier).

Allison and Burn (11) have suggested that decrement in red cell catalase or cholinesterase activity might lead to red cell disintegration; they calculated that the enzyme activity of reticulocytes was some three times that of the average cell population and that the red cell at the termination of its life span has less than 10 per cent of its enzyme activity as a reticulocyte. However, catalase is known to be present in red cells in high concentration and a change in activity of one magnitude is unlikely to be a limiting factor in protecting hemoglobin from oxidation. Further, calculations of the cholinesterase and glyoxalase activities of red cells demonstrated that the values reported in aged cells were not rate-limiting, while the suggested role of cholinesterase hydrolysis of acetylcholine providing energy for active cation transport has not been confirmed (55, 56).

The disappearance of normal cells from circulation is a linear process (57) and indicative of an intrinsic process of cell breakdown (1). During aging of the red cell, there is a diminution in cell volume, associated with decreases in water, electrolyte and dry solid (except hemoglobin) content (28). Prankerd (7) has reported a lower lipid content of bottom (old) cells compared with the top layer. The alteration in cation and water content may be a reflection of changes thus produced in the passive diffusion and active transport capacity of the lipoprotein membrane. While changes in the lipoprotein surface structure may be due to the buffeting received by red cells in circulation, aged red cells are reported to incorporate less saturated and unsaturated fatty acids from labeled acetate or glucose (58). The ultimate effect could be to render the red cells more susceptible to chemical dissolution of the red cell membrane.

SUMMARY

Erythrocytes have been separated by centrifugation into cell populations of relatively younger and older age fractions. The rate of glycolysis was less in the moiety of relatively older cells, accompanied by a lower high-energy phosphate ester content and enzyme activity (phosphohexose isomerase and aldolase). Lactic apodehydrogenase activity showed no alteration with cell aging. The aldolase concentration was a rate-limiting factor for overall glycolysis. However, hexokinase activity appeared to be the initial limitation in red cell glycolysis and may have resulted in aged cells from their decreased adenosine triphosphate content.

Cation steady states were at lower levels of active transport in old cells. Incubation without glucose produced greater deterioration of phosphate ester content for the younger cells. Refrigeration of cell fractions produced greater loss of potassium and gain of cell sodium for old cells due to lesser glycolysis; reincubation led to more

rapid recovery to normal cation contents for young cells.

Alterations in red cell glycolysis with age led to decreased available energy, producing some deterioration of active cation transport and membrane function. It is postulated that decreased glycolytic activity in the aged cell may lower the resistance of cells to destructive processes.

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A STUDY OF THE OSMOTIC BEHAVIOR OF THE HUMAN ERYTHROCYTE * †

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Despite many previous studies of the osmotic properties of erythrocytes, unequivocal answers have not been given to two important questions: *1*) Is the erythrocyte in osmotic equilibrium with its normal surrounding fluid? *2*) When the osmotic properties of the surrounding fluid are varied, does the erythrocyte gain or lose water to the extent necessary to remain in osmotic equilibrium with the new surroundings—that is, does the erythrocyte behave as a perfect osmometer over a wide range?

Previous work related to these questions has been of two general types: measurements of changes of volume of the erythrocytes as the osmotic concentration of the extracellular phase was altered; and measurements of one or more of the colligative properties of the solutions involved. The present study is of the latter type. The osmotic behavior of the normal human erythrocyte has been investigated over a wide range of concentrations by a cryoscopic method and a method for measuring the melting point of microscopic samples. The results give affirmative answers to both of the above questions and illustrate some of the difficulties which may be expected in studies of osmotic properties of any tissues.

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MATERIAL AND METHODS

All samples of blood were obtained from healthy, white male and female medical students or young physicians. Venous blood was collected anaerobically and transferred to a mercury-containing bulb, in which the blood was defibrinated by shaking. In experiments in which disruption of red cell membranes was desired, this was achieved by freezing the samples in a mixture of solid carbon dioxide and 95 per cent ethyl alcohol for five to seven minutes, followed by gradual warming to room temperature. This procedure resulted in complete hemolysis; no red cells could be identified microscopically in the samples and only an insignificant amount of red cell mass could be separated from the hemolyzed mixture by centrifugation.

In studies in which cells were separated from their surrounding fluid, this was achieved by centrifugation, under oil, at a relative centrifugal force of $600 \times G$ for 50 to 60 minutes in Tygon® tubing bent in the shape of a "U" and inserted in the cups of the centrifuge. After centrifugation, a small portion of the supernatant was reserved under oil. The tubing was then clamped a short distance below the surface of the packed cells, cut on the underside of the clamp and the cell mass expelled into an oil-containing tube. The volume of packed cells, where used, was measured in Daland tubes by centrifugation at a relative centrifugal force of $600 \times G$ for 50 to 60 minutes; this was shown to produce a constant volume. As noted in the discussion below, this measurement of volume of packed cells was considered to represent fairly accurately the cell volume in normal whole blood, but not in samples in which the osmotic activity had been significantly altered.

The first series of experiments involved measurement of freezing point depressions using a Fiske osmometer, calibrated as previously described (1). Reproducibility of the instrument on replicate samples, with care to achieve the same degree of supercooling before crystallization, was within 1 to 2 milliosmoles per Kg. water (mOsm.) throughout the range from 100 to 500 mOsm. Readings on each sample were repeated until at least two readings were within 2 mOsm. of each other; usually the first two readings were within these limits. The average of all readings is reported in the tables of Results.

The units of "milliosmoles" (per Kg. H_2O) are used in the first part of this study because the readings for freezing point depression were actually recorded in this form, because they can be readily converted to degrees

of depression of freezing point (1,000 mOsm. = freezing point depression of 1.86° C.) and because these units have become familiar as a way of expressing "osmotic activity" of biological solutions. However, the thermodynamic property of the solution which is actually being quantified by such measurements is the activity of water in the solution.

The second series of experiments involved measurements of the melting points of tiny samples of hemolyzed red cells and their former surrounding fluids. The apparatus used was made from the design of Ramsey and Brown (*2*) with slight modifications. A sample drop, about 1×10^{-6}-10^{-7} ml., is taken up in a microcapillary tube, preceded and followed by paraffin oil. It is frozen by immersion in dry-ice-alcohol for 15 to 20 seconds and placed in a bath of the apparatus which is already cooled below the melting point of the sample. With the aid of a microscopic attachment, crystals of ice can readily be seen in the sample, even in samples of pure hemolyzed red cells. The temperature of the bath is then raised very slowly, at a rate of 0.001 to 0.002° C. per minute, in the vicinity of the expected melting point. The disappearance of the last ice crystal is taken as the melting point of ice in that solution. With care and practice, replicate readings on a sample of serum agreed within less than 0.005° C. Variability in samples of hemolyzed red cells was somewhat greater but was rarely greater than 0.01° C. All readings are reported in the tables. A sample reading required 10 to 20 minutes.

TABLE I

A. Freezing points of whole blood and serum from the same samples

Sample	Whole blood	Serum	Difference
	*mOsm.**		
CL	288.8	288.0	+0.8
BL	283.5	283.5	0
PD	284.5	284.0	+0.5
ED	285.0	285.0	0
AM	289.5	289.5	0
JB	283.0	283.5	−0.5
H	282.0	282.0	0
WW	283.0	283.0	0
GN	278.0	278.5	−0.5
JM	279.5	280.5	−1.0

B. Freezing points of whole blood plus solutions of NaCl and the cell-free supernatants from the same samples

	Whole blood plus saline	Supernatant	
CL	521.0	521.5	−0.5
	228.0	228.0	0
BR	517.5	518.5	−1.0
ED	521.5	520.0	+1.5
	224.5	224.5	0
	520.5	520.0	+0.5
H	520.0	520.5	−0.5
AM	215.5	215.5	0
JB	212.5	213.0	−0.5
NH	213.0	212.5	+0.5

* In this and subsequent tables, mOsm. = milliosmoles per Kg. H_2O.

Although others (*3, 4*) have found that the freezing point of other tissues changes considerably within the first few minutes of exposure to temperatures near the freezing point, the melting point of samples of hemolyzed red cells in the present study was not detectably changed for at least one to two hours, at temperatures between 0 and 25° C., under oil. A very small change with time in the freezing point of nonhemolyzed blood was found (*vide infra*).

RESULTS

1. Freezing point depression of whole blood and serum

The finding of Hamburger (*5*) and Collins and Scott (*6*), that the depressions of freezing point of whole blood and the serum separated from the same blood are identical, was confirmed in 10 samples (Table I). This observation was extended to freezing point depressions of one-to-one mixtures of whole blood and solutions of sodium chloride, compared with the supernatant fluid removed from the same mixtures after centrifugation. In 10 such mixtures, the osmolality of which extended from 212.5 to 521.5 mOsm., the freezing point depression was the same for the whole mixture and the supernatant (Table I). In the subsequent studies to be described the freezing point depressions of samples containing *nonhemolyzed* red cells are therefore considered to be equal to the freezing point depressions of the extracellular phases of those samples.

2. Freezing point depression of whole blood before and after hemolysis

When the freezing point depressions of samples of whole blood were compared with those of the same blood after hemolysis, it was found that the osmotic activity after hemolysis was significantly lower. The mean difference in 13 samples not handled under oil was 4.46 mOsm. (Table II). The same comparison was repeated with five samples handled under paraffin oil throughout (including the determination of freezing point). A mean difference of 3.66 mOsm. was found (Table II). This mean difference was significantly different from zero but not significantly different from that of the samples not handled under oil (*7*).

In this study and in subsequent studies in which comparisons of nonhemolyzed and hemolyzed samples are made, 15 to 45 minutes elapsed

TABLE II

A. Freezing points of whole blood, unhemolyzed, and the same samples after hemolysis, not handled under paraffin oil

Sample	Unhemolyzed	Hemolyzed	Difference
	mOsm.		
WW	283.5	282.0	1.5
PE	282.5	279.5	3.0
JA	288.0	281.0	7.0
RW	288.0	281.5	6.5
AG	288.0	282.5	5.5
FW	285.5	281.5	4.0
EM	279.0	274.5	4.5
HE	285.5	282.0	3.5
S	287.0	283.0	4.0
TM	283.5	278.0	5.5
BR	283.0	279.0	4.0
CL	288.5	284.5	4.0
CP	286.0	281.0	5.0
	Mean = 285.2		Mean diff. = 4.46
			p = <.001

B. Same as A, but handled under oil throughout

FW	288.4	282.6	5.8
AT	279.5	278.0	1.5
C	285.8	281.8	4.0
TP	279.0	275.8	3.2
RC	280.8	277.0	3.8
	Mean = 282.7		Mean diff. = 3.66
			p = <.01

"t" Test of differences between the means of A and B: $t_{16\ d.f.} = 0.7686$, not significant.
"p" is the probability that the mean difference is zero (7).

between defibrination of the blood and the determination of freezing point on the hemolyzed sample. In 64 samples of various osmotic activities the freezing point of the nonhemolyzed aliquot was determined initially and again just after the determination of the hemolyzed sample. The second determination gave a slightly but significantly lower osmotic activity than the first—between 0.5 and 1.0 mOsm. lower. In the data reported in Table II and in subsequent similar tables the value for the nonhemolyzed sample is the value obtained for its freezing point immediately after the determination on the hemolyzed sample. Thus the slight change in value with time in both samples should be minimized or eliminated.

3. *Freezing point depressions of mixtures of whole blood and solutions of sodium chloride or sucrose, before and after hemolysis*

When the freezing point depressions of one-to-one mixtures of whole blood and various solutions of NaCl (5.0 Gm. per L. to slightly over 30 Gm. per L.) were compared with those of the same samples after hemolysis it was found that the osmotic activity after hemolysis was significantly lower. This difference was greater the higher the osmotic activity of the sample (Figure 1). Over the wide range of concentrations studied (208 to 747 mOsm.) the data for 83 samples not handled under oil fit the following linear equation with a high degree of significance:

$$C_H = 0.9728\ C_S + 4.09, \qquad 1)$$

where C_H = osmotic activity of hemolyzed samples (in mOsm.) and C_S = osmotic activity of unhemolyzed samples (in mOsm.). The proportion of variation explained by this equation is 0.99977 (8).

The study was repeated with 15 samples handled under oil throughout over the range 210 to 516 mOsm., with essentially the same result. Table III presents these data and is representative of the data obtained on the 83 samples not handled under oil. The data of Table III fit the following equation:

$$C_H = 0.9777\ C_S + 3.40. \qquad 2)$$

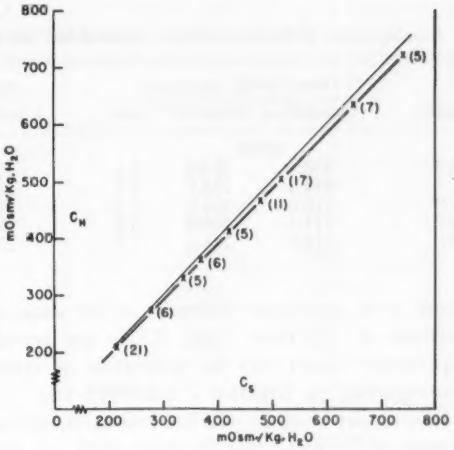

FIG. 1. THE RELATIONSHIP BETWEEN FREEZING POINT DEPRESSIONS BEFORE HEMOLYSIS (C_S) AND AFTER HEMOLYSIS (C_H) OF SAMPLES OF WHOLE BLOOD MIXED 1:1 WITH NaCl SOLUTIONS OF VARIOUS CONCENTRATIONS

The figures in parentheses are the numbers of samples at each level. The points plotted are the mean values at each level; the range is too small to depict. The thin line represents $C_H = C_S$.

TABLE III

Freezing points of blood-plus-saline, unhemolyzed, and the same samples after hemolysis, handled under oil throughout

Sample	Blood + NaCl (5.0 Gm./L.)			Blood + NaCl (9.0 Gm./L.)			Blood + NaCl (22.22 Gm./L.)		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*			*mOsm.*		
AT	210.1	209.5	0.6	278.5	276.0	2.5	510.7	503.9	6.8
C	214.4	213.1	1.1	282.3	279.0	3.3	515.9	506.5	9.4
TP	210.6	208.9	1.7	279.0	276.0	3.0	509.5	500.7	8.8
B	217.4	216.6	0.8	284.5	282.3	2.2	516.2	508.2	8.0
RC	210.4	208.6	1.8	279.8	275.8	4.0	509.2	502.2	7.0

There is no significant difference in the slope or intercept of Equations 1 and 2. In the second experiment (under oil) the proportion of variation explained by Equation 2 is 0.99995 (8).

In a similar study in which solutions of sucrose instead of sodium chloride were used, at two levels of osmotic activity of the mixture (217 and 497 mOsm.), a comparable decrease in activity with hemolysis was found (Table IV).

4. The effects of addition of dry urea

Samples of whole blood were mixed one-to-one with a solution of NaCl, 5.0 Gm. per L., used previously to produce a hypotonic mixture (about 212 mOsm.). Various amounts of dry urea were then added to the mixture, the amounts so chosen that the whole range of osmolalities of the saline studies given above was reproduced with the urea-containing mixture. Freezing point depression was then measured with and without hemolysis. The results are given in Table V. These data indicate that, in contrast to the results with saline and sucrose, the differences obtained between unhemolyzed and hemolyzed samples are not significantly altered by the addition of varying quantities of urea. The average difference of similar samples without urea added was 1.73 mOsm.; the average difference of these containing urea is 2.09 mOsm.

Comments on Studies 2, 3 and 4. The osmotic activity—freezing point depression or any other measure of colligative properties—of a sample of blood would be expected to be the same after rupture of the red cell membranes as it was before rupture if: *1*) the red cell contents before rupture were in osmotic equilibrium with their surroundings; *2*) with rupture the mixing of the two previously separate solutions did not change appreciably the activity coefficients of the various solutes present; and *3*) no solutes or water were added to or lost from the solution with rupture. Since the present studies show a definite change in osmotic activity of the mixture after hemolysis, at least one of the above conditions does not hold. The change in osmotic activity with hemolysis is small but, within the limits of the design of the

TABLE IV

Freezing point of blood-plus-sucrose, unhemolyzed, and the same samples after hemolysis

Sample	Blood + sucrose (53.1 Gm./L.)			Blood + sucrose (243.9 Gm./L.)		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*		
D-1	218.5	216.5	2.0	498.0	488.0	10.0
D-2	218.5	217.5	1.0	501.0	489.5	11.5
D-3	221.5	221.0	0.5	501.5	489.5	12.0
D-4	214.5	212.5	2.0	491.5	485.5	6.0
D-5	215.0	215.0	0	496.0	488.0	8.0
D-6	216.0	213.5	2.5	500.0	484.0	16.0
D-7	217.5	217.0	0.5	495.0	486.5	8.5
D-8	216.0	213.0	3.0	497.0	486.0	11.0
D-9	217.0	214.0	3.0	489.0	479.5	9.5
D-10	213.5	212.5	1.0	496.0	482.0	14.0
		Mean diff. = 1.55			Mean diff. = 10.65	

TABLE V

*Freezing points before and after hemolysis of samples to which dry urea was added**

Sample	14 mg. urea added			31 mg. urea added		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*		
4G	278.5	276.5	2.0	346.5	344.0	2.5
5G	277.0	276.0	1.0	345.0	343.0	2.0
6G	271.5	270.5	1.0			
7G				355.0	354.0	1.0
8G						
9G				353.0	350.0	3.0
10G						
11G	268.5	266.0	2.5	349.5	347.5	2.0
12G	269.0	267.0	2.0	349.5	347.0	2.5
13G	270.5	267.5	3.0	345.5	343.0	2.5
14G	279.0	276.0	3.0	348.5	347.0	1.5
15G	272.5	271.5	1.0			
		Mean diff. = 1.94			Mean diff. = 2.13	
	48 mg. urea added			64.5 mg. urea added		
4G	426.5	425.0	1.5	497.5	492.5	5.0
5G	426.5	426.5	0			
6G	418.0	415.5	2.5	510.5	504.5	6.0
7G				505.0	499.5	5.5
8G				492.5	491.5	1.0
9G				504.5	502.0	2.5
10G				506.5	505.0	1.5
11G	421.0	421.0	0	494.0	494.0	0
12G	420.5	419.0	1.5			
13G	419.0	415.5	3.5			
14G	421.0	420.5	0.5	495.0	494.0	1.0
15G	418.0	417.0	1.0	484.5	481.0	3.5
		Mean diff. = 1.31			Mean diff. = 2.89	

* Each sample contained 2 ml. blood plus 2 ml. NaCl solution, 5.0 Gm. per L.

present study, highly predictable from sample to sample of normal blood. Furthermore, the difference between hemolyzed and unhemolyzed aliquots of the same sample varies in a predictable fashion as the osmotic concentration of the fluid outside the red cells is varied by changing the concentrations of solutes which will not readily cross the red cell membranes, *e.g.*, sodium chloride or sucrose. However, this difference is not affected by the addition of urea, a solute which diffuses easily into the red cell. Thus, the *total* osmotic activity of the solutions inside and outside the cell is not the determinant of the variation in differences of osmotic activity before and after hemolysis.

These results are consistent with, but more extensive than, previously reported studies. Thus Hamburger (9) found that the freezing point depression of horse and dog serum was 1 to 2 per cent lower than that of the hemolyzed blood. Mukai (10) obtained similar results in samples handled under paraffin oil, with a mean difference between serum and hemolyzed whole blood (expressed in the units of the present paper) of 5.9 mOsm. Utilizing Barger's vapor pressure method, which involves the measurement of change of length of drops of fluid, separated by air spaces in capillary tubes, Mukai found no measurable difference in the vapor pressures of a mixture of laked blood and serum (1:1), and serum alone. However, in the diluted blood samples with which he was working, a difference in vapor pressure between serum and hemolysate-serum mixture of a magnitude similar to that observed in the present study could have been missed.

Of the three conditions mentioned above, there is independent evidence that the second, absence of appreciable change in activity coefficients of the solutes present, does not hold. In his studies of the osmotic properties of hemoglobin, Adair (11) found that the activity coefficient of hemoglobin increased with increasing concentration of

hemoglobin; *i.e.*, the total osmotic activity of hemoglobin solutions increased at a progressively greater rate than the concentration of hemoglobin in the solution. Similar properties for plasma proteins have been described by Scatchard, Batchelder and Brown (12). From Adair's findings one would predict the following events: *1*) When whole blood is hemolyzed, and the *concentration* of hemoglobin in the solution falls from that of the interior of the red cell to a much lower level in the hemolyzed blood, the *osmotic activity* of the hemoglobin would decline even more. Thus the total osmotic activity of the hemolyzed blood would be expected to have a lower value than the activity produced by the same total amount of solutes in the same total amount of water when the hemoglobin was contained in the red cell. *2*) Similarly, when the blood is mixed with hypertonic NaCl, if water moves freely across the cell membrane in response to osmotic forces, water would leave the cell, and the concentration of hemoglobin would rise. However, the osmotic activity of hemoglobin would rise even more and thus osmotic equilibrium would be reached with less movement of water out of the cell than would have occurred if there were no change in the activity coefficient of the hemoglobin. When such cells are then hemolyzed, the concentration of hemoglobin falls more than it does when cells in isotonic media are hemolyzed, and the osmotic activity of the hemoglobin would be expected to decline even more. Thus a greater difference in osmotic activities, before and after hemolysis, would be expected under hypertonic conditions than under isotonic conditions. The reverse would be expected with cells exposed to hypotonic media.

Qualitatively, the present data are in agreement with such predictions based on Adair's findings. An approximate quantitative comparison can be made for the hemolysis of normal whole blood. For an average concentration of hemoglobin within the normal red cell of 34 Gm. per 100 ml. solution, Adair's data indicate that the osmotic activity of the hemoglobin would be about 18.7 mOsm. If we assume that the total osmolality within the cell equals that of the serum, which has a mean value in our studies of 285.3 mOsm., then the osmotic activity of intracellular solutes other than hemoglobin would be: $285.3 - 18.7 = 266.6$ mOsm. With hemolysis, the hemoglobin would be diluted to a concentration of 15.4 Gm. per 100 ml. solution. At this concentration the hemoglobin would have, by Adair's data, an osmotic activity of 3.7 mOsm.

The average contents of water measured in 10 samples of blood (together with packed cell volume) and five samples of serum were 0.3145 ml. H_2O in the red cell per ml. blood and 0.5092 ml. H_2O in the serum per ml. blood.

A prediction of the total osmotic activity of the hemolyzed sample can be made with the above data by the following calculations, if one assumes that the activity coefficients of other solutes in red cell and serum will change relatively little when the two solutions are mixed:

$$\text{Predicted osmotic activity after hemolysis} = \frac{\left(\begin{matrix}\text{H}_2\text{O content} \\ \text{of serum}\end{matrix} \times \begin{matrix}\text{osmotic activity} \\ \text{of serum}\end{matrix}\right) + \left(\begin{matrix}\text{H}_2\text{O content} \\ \text{of cells}\end{matrix} \times \begin{matrix}\text{osmotic activity of cells} \\ \text{other than hemoglobin}\end{matrix}\right)}{\text{total H}_2\text{O content of 1 ml. blood}} + 3.7$$

$$= \frac{(0.5092 \times 285.3) + (0.3145 \times 266.6)}{0.8237} + 3.7$$

$$= 281.8 \text{ mOsm.}$$

Therefore, the predicted decrease in osmotic activity of normal whole blood upon hemolysis is: $285.3 - 281.8 = 3.5$ mOsm. This compares well with the average values found in the present studies of a decrease of 3.7 mOsm. in samples handled under oil, or 4.5 mOsm. without oil.

Thus the change in osmotic activity which occurs with hemolysis of red cells in whole blood *could* be accounted for by the change in the activity coefficient of the hemoglobin alone. Similar calculations have not been made for the series of samples of blood-plus-saline before and after hemolysis at different concentrations because of the unreliable nature of measurements of packed cell

volume of red cells exposed to hypotonic or hypertonic media (13). A reliable value for packed cell volume is required for the calculation of the concentration of hemoglobin in the unhemolyzed cells, from which its osmotic activity may be determined from Adair's data. However, the differences in osmotic activity seen in the various blood-saline mixtures, before and after hemolysis, are in the direction which is to be expected from Adair's observations.

It may be noted that the apparent loss of osmotic activity found by Appelboom, Brodsky, Tuttle and Diamond (14) for boiled whole blood might also be accounted for by the change in the activity coefficient for hemoglobin introduced by dilution of their samples.

The fact that the changing activity coefficient of hemoglobin can account for the change in osmotic activity with hemolysis does not, of course, exclude other possible explanations of the observed change. A more direct comparison of the osmotic activity of the pure contents of the red cells with that of their surroundings is highly desirable. In the present studies attempts to determine directly, with the Fiske osmometer, the freezing point of pure hemolyzed red cells did not give satisfactory results because of the great and irregular degree of supercooling. Previous writers have recognized supercooling as a problem (15, 16). The wide range of values obtained in our attempts, similar to the range reported by Brodsky and co-workers (4), for crushed red cells from normal dogs, could not be considered satisfactory direct measures of the osmotic activity of ruptured red cells.

Attempts to compare the osmotic activity of hemolyzed red cells with that of their surroundings by a comparative vapor pressure technique [the isopiestic technique (17)] were also unsatisfactory. Details of the problems encountered will be furnished upon request.

A third method of comparing the colligative properties of these solutions, the use of melting points, gave more satisfactory results, as follows.

5. *Melting points of hemolyzed red cells and their former surrounding fluid*

Three types of samples were used: *1*) whole blood mixed one-to-one with a sodium chloride solution, 5.0 Gm. per L., and then separated into cells and supernatant; *2*) whole blood mixed one-to-one with sodium chloride solution, *22.22* Gm. per L., and then separated into cells and supernatant; and *3*) whole blood separated into cells and serum. Separation of cells, hemolysis of the red cells and measurement of melting points on microscopic samples were done as described in Methods.

Tables VI, VII and VIII give the results. By an analysis of variance of these data one can place the limits of confidence given in the tables about the mean differences between melting points of hemolyzed red cells and the surrounding fluid to which they had been exposed prior to hemolysis. Thus, as shown in Table VI for red cells exposed to a hypotonic solution, the probability is 0.95 that the narrow range, +0.0062 to −0.0017° C. (+3.32 to −0.91 mOsm.), includes the true value for the mean difference between surrounding solutions and red cell contents. This range includes zero difference; *i.e.*, the mean difference in melting point is not significantly different from zero. The same type of statement can be made from Table VII, for red cells exposed to a hypertonic solution, where the probability is 0.95 that the range, +0.0054 to −0.013° C. (+2.90 to −6.81 mOsm.), includes the true value for the mean difference, and from Table VIII for red cells of normal whole blood, where the probability is 0.95 that the range, +0.0004 to −0.0067° C. (+0.19 to −3.57 mOsm.), includes the true value for the mean

TABLE VI

Melting points of components of samples of whole blood mixed 1:1 with NaCl solution, 5.0 Gm. per L.

Sample	1 Melting point of supernatant	2 Melting point of hemolyzed red cells
	°C.	°C.
NH	−0.401, 0.400	−0.411, 0.408
AM	−0.401, 0.403	−0.400, 0.404
BW	−0.410, 0.404	−0.410, 0.409
MS	−0.406, 0.405	−0.405, 0.402
WT	−0.410, 0.410	−0.410, 0.412
WT	−0.410, 0.410	−0.413, 0.413

Mean difference, melting point of supernatant minus melting point of hemolyzed red cells = +0.0023° C. (+1.23 mOsm.); 95% confidence limits about the mean difference = +0.0062° C. to −0.0017° C. or +3.32 mOsm. to −0.91 mOsm.

TABLE VII

Melting points of components of whole blood mixed 1:1 with NaCl solution, 22.2 Gm. per L.

Sample	1 Melting point of supernatant	2 Melting point of hemolyzed red cells
	°C.	°C.
MT	−1.010, 1.002, −1.002, 0.998, 1.000	−0.980, 0.995, 0.985, −0.980, 0.988, 0.995
AL	−1.020, 1.022, −1.030, 1.032	−1.040, 1.050, 1.035
EO	−1.018, 1.018	−1.030, 1.040, 1.020
BW	−1.020, 1.025	−1.015, 1.025, 1.030
CM	−1.010, 1.010	−1.012, 1.012
LH	−1.015, 1.014	−1.002, 0.995, 0.995
DK	−1.012, 1.008	−1.008, 1.005
AB	−1.005, 0.998	−0.998, 1.003
TF	−1.040, 1.040	−1.020, 1.030
TD	−1.026, 1.030	−1.022, 1.020

Mean difference, melting point of supernatant minus melting point of hemolyzed red cells = −0.0034° C. (−1.82 mOsm.); 95% confidence limits about the mean difference = +0.0054° C. to −0.013° C. or +2.90 mOsm. to −6.81 mOsm.

difference between hemolyzed red cells and serum.

The studies on whole blood (Table VIII) are presented in two parts. Series A includes the first samples studied by this method. Statistical analysis of the first 10 samples indicated a difference in melting point between serum and cells, that of the cells being slightly higher (*i.e.*, less solute concentration). However, it was noted that this difference was contributed almost entirely by the differences in the first three samples that had been measured. Accordingly, another series of whole blood samples was studied, with two additional precautions: The samples were centrifuged in a temperature controlled centrifuge at 22° C., and the order in which the two readings on serum and two readings on red cells were done was randomized for the total of four readings on each sample. The control of temperature of centrifugation was instituted because it had been noted that in a noncooled centrifuge the samples became quite warm by the end of centrifugation.

The results of measurements on whole blood with these added precautions and at a time when technical handling of the whole procedure was improved by practice are given in Series B of Table VIII. Variability within samples was decreased and there is clearly no significant difference in this series in the melting point of hemolyzed red cells and serum from the same samples. The 95 per cent confidence limits on the mean difference of + 0.00042° C. are from −0.00712 to + 0.00795° C. (+ 4.26 to − 3.82 mOsm.), distributed almost equally about zero difference. As already noted, if all determinations on normal whole blood are included in one analysis, the mean difference is also not significantly different from zero.

Thus there appears to be no significant difference between the melting points of hemolyzed red cells and the fluid to which the cells had been exposed, prior to separation and hemolysis, at the three levels of osmotic activity studied.

DISCUSSION

In the few previous studies of osmotic properties of red cells which have utilized direct measure-

TABLE VIII

Melting points of components of whole blood

Sample	Melting point of serum	Melting point of hemolyzed red cells
	°C.	°C.
A. First series		
HO	−0.547, 0.547	−0.530, 0.535, 0.540
CM	−0.540, 0.542	−0.531, 0.530, 0.528
LH	−0.540, 0.540	−0.533, 0.525
DK	−0.535, 0.542	−0.540, 0.538
AB	−0.535, 0.535	−0.530, 0.531
TF	−0.530, 0.530	−0.528, 0.533
TD	−0.540, 0.545	−0.532, 0.532
NH	−0.517, 0.523	−0.518, 0.525
AM	−0.530, 0.531	−0.530, 0.528
BW	−0.540, 0.539	−0.537, 0.533

Mean difference, melting point of serum minus melting point of hemolyzed red cells = −0.0053° C. (−2.84 mOsm.); 95% confidence limits about the mean difference = −0.0014° C. to −0.0092° C. or −0.73 mOsm. to −4.95 mOsm.

B. Second series		
SF	−0.567, 0.561	−0.572, 0.576
JM	−0.530, 0.530	−0.530, 0.533
TF	−0.540, 0.535	−0.522, 0.532
CG	−0.530, 0.530	−0.536, 0.535
NC	−0.519, 0.520	−0.520, 0.519
RS	−0.529, 0.529	−0.527, 0.523

Mean difference, melting point of serum minus melting point of hemolyzed red cells = +0.00042° C. (0.23 mOsm.); 95% confidence limits about the mean difference = +0.0080° C. to −0.0071° C. or +4.26 mOsm. to −3.82 mOsm.

For Table VIII treated as a unit, the mean difference = −0.0032° C. (−1.72 mOsm.); 95% confidence limits about the mean difference = +0.00036° C. to −0.0067° C. or +0.19 mOsm. to −3.57 mOsm.

ment of a colligative property of the red cell contents, the data have been in general agreement with the hypothesis that the red cell is in osmotic equilibrium with its surroundings. Hill (18) employed a thermo-electric method for the determination of vapor pressures and obtained similar values for the serum and hemolyzed red cells from one sample of slaughterhouse blood. Roepke and Baldes (19) also used a thermo-electric method for measuring vapor pressures, and found the values for lysed human red cells to be very nearly equal to those of the intact cells (*i.e.*, the trapped extracellular fluid). When human cells were washed with hypertonic solutions of NaCl or sucrose, they found a decrease in vapor pressure after hemolysis, but this they attributed to dilution by trapped extracellular fluid. They recognized that such dilution would change the osmotic coefficient of hemoglobin.

The present experiments with melting point determinations demonstrate, with considerable statistical confidence, that the osmotic activity of the contents of the red cell is not significantly different from that of the extracellular phase to which the cell was exposed prior to rupture. This is true not only of cells previously in normal serum, but of cells which had been exposed to extracellular mixtures of serum and saline at concentrations from 210 to 520 mOsm. The red cell therefore appears to act as a "perfect osmometer" over the range of concentration studied here, gaining or losing sufficient water to remain in osmotic equilibrium with its surroundings. Stated in other terms, the activity of water inside the red cell is the same as that outside over a wide range of activities. Two untested assumptions underlie this statement: *1*) The actual process of rupture of the red cell membrane by freezing and thawing does not significantly alter the solute concentration in the red cell contents; and *2*) measurements made at -0.3 to $-1.0°$ C. accurately reflect the comparative colligative properties of red cells and surroundings at room temperature or body temperature. This assumption is supported by the finding of Roepke and Baldes (19) that there is no significant effect of temperature, from 0 to 29.4° C., on the comparative vapor pressures of red cells and reference solutions of serum, sodium chloride or sucrose.

In contrast to the above results and interpretation of studies of colligative properties, the studies of the osmotic behavior of the red cell using measurement of changes of cell volume have been generally interpreted as indicating that the cell is not a perfect osmometer [see Ponder (20, 21) for reference to the early literature and for discussion of these findings and of the sources of error in measuring cell volumes]. In most such experiments the changes in cell volume when the osmotic activity of the extracellular fluid is changed have not been as great as would be predicted for a simple osmometer. Even though a few studies, such as that by Hendry (13), give opposite results, the general consistency of direction of change in the data obtained by various methods of measuring cell volume makes it necessary to seek some explanation for the less than expected changes in volume with changes in external osmotic activity.

Four explanations have been proposed: *1*) The structure of the membrane resists change in volume. This view would allow the red cell to be in osmotic equilibrium with its surroundings in isotonic media but not in hypotonic or hypertonic media, which is not consistent with the findings of the present study.

2) The red cell may gain or lose solutes as the composition of the surrounding media is changed. The present studies have no direct bearing upon such possible movements. However, the results obtained with the addition of sucrose solutions (Table IV) and urea (Table V) indicate that the osmotic behavior of the red cell is related to the concentrations of solutes which do *not* cross the red cell membrane.

3) Some of the water within the red cell is "bound", *i.e.*, not available to participate in changes in the red cell content of water in response to changes in osmotic activity outside the cell.

4) The changes in cell volume are what would be expected on the basis of Adair's evidence (11) for the changing osmotic coefficient of hemoglobin, and with allowance for this property of hemoglobin the red cell does behave as a perfect osmometer.

The acceptability of the "bound" water concept would seem to depend upon the sense in which this term is used. It can be taken to mean the same thing as is meant by the changing osmotic

coefficient of hemoglobin. That is, when one states that the osmotic coefficient of hemoglobin increases with increasing concentration of hemoglobin in a solution, an equivalent statement would be that the activity coefficient (and therefore total activity) of water decreases under these circumstances. If "bound" water is equated with "decreased activity coefficient" of water, then explanations 3 and 4 are equivalent and are consistent with available evidence including the present study.

Another approach to the question of "bound" water has been to add known amounts of solutes which would be expected to distribute in all available water which could act as "solvent" water and then to measure the concentration of the solute in the system or its effect on the colligative properties of the system. Earlier studies indicated the presence of a significant proportion of "nonsolvent" water in the red cell (*22*, *23*). Hill (18) found less than 5 per cent of red cell water to be nonsolvent water. Roepke and Baldes (19) found small but consistent amounts of "nonsolvent" water which increased as the nondiffusible solute concentration of the extracellular phase was raised. They believed that the changing osmotic coefficient of hemoglobin described by Adair (11) could account for most of the "anomalous osmotic behavior" of red cells, but that some of the water of cells was not solvent for added crystalloids. However, Hutchinson (*24*), using $C^{14}H_3OH$, C^{14}-n-butyl alcohol and H_2O^{18}, found that these molecules reached rapid equilibrium with the contents of human red cells and that all of the water of the red cells acted as "free" water with respect to these substances. This is probably the most refined technique that has been applied to the measurement of "free" water. MacLeod and Ponder (*25*) have pointed out that "nonsolvent" water and "bound" water may not be identical.

Dick and Lowenstein (*26*) recently measured the change in red cell volume in the hypotonic range by a technique of immersion refractometry. Their data, which differed from that of the earlier literature in that they were so gathered that statistical treatment was possible, were consistent with the hypothesis that the red cell is a "perfect osmometer" if due allowance is made for Adair's evidence on the physicochemical properties of hemoglobin. The same interpretation fits the present data on the decrease in osmotic activity of whole blood, blood-plus-saline and blood-plus-sucrose solution. Thus it may be said that evidence from virtually all studies of the osmotic behavior of the red cell, when considered in the light of the known properties of hemoglobin, is consistent with the view that the red cell is in osmotic equilibrium with its surroundings over a wide range of concentrations.

Two observations about the method of conducting studies of the colligative properties of biological fluids have emerged from these experiments. First, examination of colligative properties of other types of cells will probably require a technique by which the cells may be effectively separated from the extracellular fluid prior to rupture of the cell membranes. It appears likely that the type of relationship which exists between the osmotic coefficient of hemoglobin and its concentration also applies to the proteins of other cells. Dick (*27*) comments on the probable importance of the changing osmotic coefficients of intracellular proteins in his study of the osmotic properties of fibroblasts.

Second, the problem of supercooling of fluids containing a high proportion of nonaqueous substances, which prevented accurate determination of the freezing point of hemolyzed red cells in this study, would likely be true of any other intracellular fluid. Others have reported a great variability among determinations of this type (4). Other ways of measuring colligative properties, such as the melting point technique used in this study or that described by Maffly and Leaf (*28*), would probably be a sounder approach.

SUMMMARY

The osmotic behavior of the normal human red cell has been studied by two techniques which measure colligative properties. In all studies the number of samples was large enough to allow a statistical statement about the limits of confidence of the results.

The freezing point depressions of whole blood and serum from the same samples were found to be identical; this was also true when saline solutions of various concentrations were mixed with the whole blood. The freezing point depression of whole blood, whole blood-plus-saline or whole blood-plus-sucrose solutions, was found to be less

after hemolysis than before hemolysis. Such a difference was present over a wide range of osmotic concentrations and varied directly with the concentration in a highly predictable fashion. These data are consistent with Adair's observation (11) that the osmotic coefficient of hemoglobin varies in a curvilinear fashion with its concentration in solution. These findings are therefore compatible with the hypothesis that the red cell remains in osmotic equilibrium with its surrounding fluid. Most previous studies which bear on this point may be similarly interpreted.

Attempts to measure the freezing point depression of hemolyzed red cells alone were not satisfactory because of the problem of supercooling. Attempts to measure the relative vapor pressures of hemolyzed red cells and serum by the isopiestic technique were also not successful.

By use of a technique for measurement of melting points of microscopic samples, it proved possible to determine this property of hemolyzed red cells and the extracellular fluid to which they had been exposed before hemolysis. Over a range of concentrations from 210 to 520 milliosmoles per Kg. H_2O, the melting points of the intracellular and extracellular phases were found to be not significantly different.

It is concluded that the normal human red cell is in osmotic equilibrium with its surrounding fluid and that this equilibrium is maintained over a wide range of concentrations.

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INCORPORATION OF RADIOIRON INTO MYOGLOBIN IN AN *IN VITRO* SYSTEM *

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It is known that avian erythrocytes (1, 2), bone marrow (3), liver, muscle and kidney (4) are capable of synthesizing heme *in vitro*. Glycine, δ-aminolevulinic acid, porphobilinogen and protoporphyrin (2, 5–7) are among the known intermediates in this process. Per unit weight, muscle is one of the least active tissues in the synthesis of heme, and few studies have been made of the process in this tissue.

Previously (8, 9), we have described procedures for the isolation of myoglobin which were found suitable for the preparation of this protein for physical characterization. In order to gain further information about the metabolism of myoglobin, these procedures for myoglobin isolation have been applied to a study of radioiron incorporation into myoglobin in dog and human muscle. In these studies, the myoglobin heme was labeled with Fe^{59} in a system similar to that described by others for avian erythrocytes (2). By this means it has been possible to demonstrate that extracts of dog and human muscle can use radioiron for the formation of myoglobin. It appears that heme synthesis in muscle is similar in many ways to that in other tissues.

METHODS

Preparation of extracts and incubation mixture. Mongrel dogs were sacrificed by the intracardiac injection of 10 per cent pentobarbital. Three hundred to 500 Gm. of muscle was dissected from the hind limbs, trimmed free of fat and placed in a beaker over ice. The muscle was ground twice in an electric meat grinder. The brei was mixed with an equal volume of 0.15 M potassium chloride containing approximately 20 mg. each of penicillin and streptomycin and allowed to stand for 12 to 18 hours. Shorter extraction periods were associated with less iron incorporating activity in the final extracts. Sodium fluoride, 0.1 M, a concentration which has been shown to have no effect on heme synthesis in avian erythrocytes (10), was added to the extracting fluid to inhibit glycolysis. This mixture was homogenized in a Waring blendor for three minutes. All of these procedures were carried out at 4° C. Centrifugation at 1,500 rpm for 30 minutes (500 × G) then was carried out at room temperature.

The incubation mixtures were prepared in 50 ml. Erlenmeyer flasks by adding the following: 14 ml. of the supernatant fluid from the dog muscle homogenate; 4 ml. of Tris [Tris(hydroxymethyl) aminomethane] buffer (final concentration 0.5 M Tris) at pH 7.7; 1 ml. of ferrous^{59} citrate (1.0 to 2.0 μc., specific activity 1 to 3 mc. per mg. Fe); and either 1 ml. of 0.9 per cent sodium chloride or other additive according to the experimental protocol. All incubations were carried out in air in a Dubnoff shaker at 37° C., 70 cpm, for four hours. Further radioiron incorporation was then stopped by the initial step in the myoglobin isolation procedure, the addition of one-fourth the reaction volume of saturated basic lead acetate.

Myoglobin was isolated by differential solubility in phosphate buffer as described previously (8, 9). In order to avoid contamination of the myoglobin with radioactive iron phosphate complexes and to concentrate the myoglobin for counting, a special procedure was used after the hemoglobin had been precipitated with concentrated phosphate buffer. The samples were shaken with 10 ml. of chloroform for 20 minutes in a Kahn shaker. This precipitated the myoglobin at the interphase as a fluffy, readily dispersed precipitate. After the supernatant solution had been discarded, the myoglobin layer was punctured with a wooden rod to allow egress for the chloroform. The myoglobin precipitate was then washed six times with 1 per cent trichloroacetic acid. The final wash was nonradioactive and contained less than 0.5 μg. per 100 ml. of phosphate. After carrying out the last wash in a counting vial, the radioactivity of the samples was measured in a well-type scintillation counter.

Since the interpretation of the results to be presented is dependent on the demonstration that myoglobin prepared by this method is, indeed, essentially free of hemoglobin, several experiments were carried out to demonstrate this fact. Some of these studies have been presented previously (9).

By measuring optical density of cyanide-treated dog

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TABLE I

Demonstration of removal of hemoglobin from myoglobin in dog muscle extracts *

Sample	Hemoglobin added	Total hemoglobin	cpm added	Myoglobin isolated	Counting rates myoglobin samples cpm			Hemoglobin remaining	Hemoglobin remaining	Myoglobin in final samples
					Total	Net	S			
	mg.	*mg./sample*		*mg.*				%	*mg.*	%
1	0	25	0	19.0	163	0				
2	0	25	0	20.0	163	0				
3	27	52	1,944	18.8	172	8	±2.3	0.47	0.24	98.7
4	27	52	1,944	18.7	181	17	±2.3	0.87	0.45	97.6
5	54	79	3,888	19.4	194	30	±2.4	0.77	0.61	96.9
6	54	79	3,888	17.6	190	26	±2.4	0.67	0.52	97.0
7	135	160	9,720	18.9	225	61	±2.6	0.63	1.0	94.7
8	135	160	9,720	17.5	216	52	±2.6	0.53	0.85	95.1
9	270	295	19,440	19.2	211	47	±2.5	0.24	0.71	96.3
10	270	295	19,440	20.0	213	49	±2.5	0.25	0.74	96.3

* Samples were composed of 16 ml. crude muscle extract and 2.5 ml. water or hemoglobin solution. All samples were counted for 16,384 counts. Hemoglobin specific activity = 72 cpm per mg.; hemoglobin content of crude extract before additions = 25 mg. per 16 ml.

muscle extract at 540 mμ before and after the addition of phosphate buffer, an estimate of the relative amounts of hemoglobin and myoglobin in the extract was obtained. These values ranged from 5 to 25 mg. of hemoglobin and 18 to 36 mg. of myoglobin per 16 ml. of extract. Accordingly, radioactive hemoglobin, prepared by aqueous hemolysis of triply-washed erythrocytes from a dog previously given intravenous ferrous[59] citrate was added to identical 16 ml. samples of muscle extract prepared from another dog. The amount of hemoglobin added varied from one to 10 times the hemoglobin content of the crude extract. Myoglobin was isolated from the extracts and the radioactivity measured in the scintillation counter. The results are shown in Table I. Because the levels of radioactivity remaining in the samples were so low, long counting times were used, and the standard deviation of each sample counting rate was calculated according to the formula:

$$S = \pm\sqrt{\frac{s+b}{t_s} + \frac{b}{t_b}}$$

where S = standard deviation of the sample counting rate; s = sample counting rate; b = background counting rate; t_s = total time sample counted; and t_b = total time background counted. These results are included in Table I.

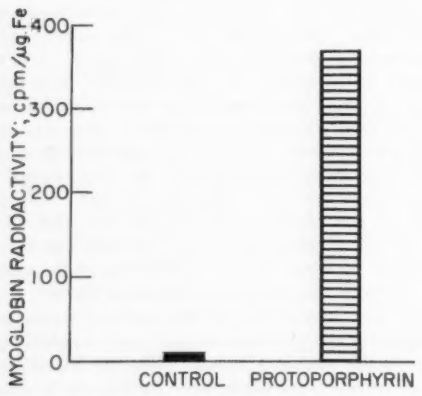

FIG. 1. THE EFFECT OF PROTOPORPHYRIN ON THE INCORPORATION OF RADIOIRON INTO MYOGLOBIN IN A TYPICAL EXPERIMENT

In this experiment, myoglobin purity with reference to hemoglobin ranged from 94.7 to 98.7 per cent. In particular, at levels of hemoglobin contamination closest to the hemoglobin level of the crude extract used in the studies to be presented, the final myoglobin samples contained 97.6 to 98.7 per cent myoglobin. This experiment demonstrated effective removal of hemoglobin and secondarily showed that elaborate procedures to perfuse muscles free of blood prior to extraction were unnecessary.

After counting, the myoglobin was washed into Kjeldahl flasks for digestion and the iron content was determined by the method of Gubler and co-workers (11). Radioactivity has been expressed as cpm per μg. of myoglobin iron. It should be noted that no attempt has been made to control the total iron content of each flask. This, coupled with the innate variations in the system from dog to dog, prevents comparison between absolute values of radioactivity from experiment to experiment. In order to obviate this difficulty, internal controls, in the form of extracts containing buffer and radioiron, but without protoporphyrin or other additives, have been included in each experiment.

Techniques similar to those employed in the dog studies were used in the study of human muscle. This was obtained at autopsy from two nondystrophic human subjects and two patients with myotonic dystrophy.

Protoporphyrin-9-methyl ester [1] was prepared by hydrolysis in 7 N hydrochloric acid for 18 hours, followed

[1] Obtained from Monadnock Research Institution, Antrim, New Hampshire.

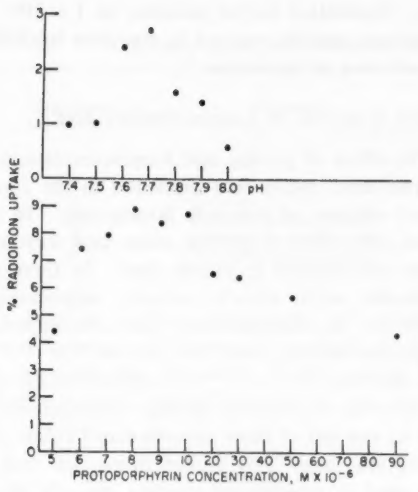

FIG. 2. THE EFFECTS OF HYDROGEN ION AND OF PROTOPORPHYRIN CONCENTRATION ON RADIOIRON UPTAKE INTO MYOGLOBIN

by removal of excess acid in a desiccator over sodium hydroxide. The protoporphyrin hydrochloride was dissolved in 0.14 M sodium bicarbonate prior to addition to the incubation flasks. Glycine and δ-aminolevulinic acid were dissolved in water and neutralized with sodium hydroxide prior to addition to the incubation flasks. In some experiments, heme was isolated from the reaction mixtures by the method of Fischer (12) and recrystallized by the method of Shemin, London and Rittenberg (13). The radioactivity of the heme then was determined. In these experiments, the results were expressed as the percentage of added radioiron which was incorporated into heme (per cent uptake of Fe^{59}) (2).

RESULTS

Effect of protoporphyrin

The addition of protoporphyrin to flasks incubated four hours resulted in a 10- to 200-fold augmentation of radioiron uptake. Zero time controls, containing protoporphyrin but not incubated, yielded nonradioactive myoglobin. A typical experiment is shown in Figure 1. In order to demonstrate whether the radioactivity of the myoglobin was due to the incorporation of Fe^{59} into the heme or could be attributed to nonspecific binding, the myoglobin from a reaction mixture was purified, the heme of the myoglobin was isolated and the radioactivity of the heme was determined. Of the radioiron present in the myoglobin, 95 per cent was recovered in the heme.

The effects of protoporphyrin concentration and of pH are shown in Figure 2. The optimal protoporphyrin concentration was 8×10^{-6} M to 1×10^{-5} M, and the optimal pH was 7.6 to 7.7. Therefore, in subsequent studies, the assays were performed at pH 7.6 to 7.7 and 9×10^{-6} M protoporphyrin was used.

Effect of time of incubation

Myoglobin isolated from samples after incubation times varying from 15 to 240 minutes showed rapid increase in radioactivity in the first 15 to 30 minutes (Figure 3) followed by a more gradual rise thereafter. Incorporation of radioactive iron into the myoglobin heme was still continuing at four hours.

Effect of heat

Since heating the muscle extract at 70° C. for 15 minutes prior to assay altered the properties of the myoglobin to such an extent that good recoveries could not be obtained, heme isolation was used to study the effect of heat on this system. Since the heme was isolated from whole extracts, rather than from myoglobin itself, these results apply to overall heme synthesis in muscle extracts and not necessarily to myoglobin heme, *per se*. Under these conditions, heme synthesis was inhibited 62 per cent as compared with control unheated extracts.

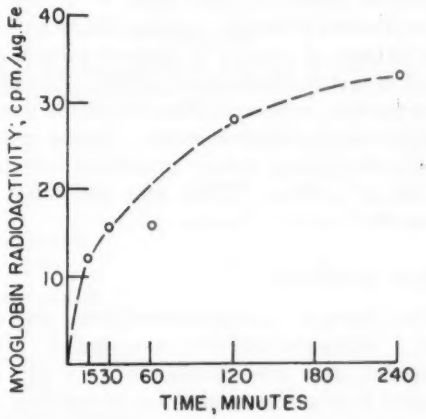

FIG. 3. THE EFFECT OF TIME ON THE INCORPORATION OF RADIOIRON INTO MYOGLOBIN

TABLE II

Effect of ultracentrifugation of muscle homogenate supernate on radioiron uptake into heme

Sample	Uptake
	%
Protoporphyrin + Fe control	0.5
Whole (initial) extract	10.2
Supernatant solution	2.4
Residue	11.0

Effect of ultracentrifugation

A muscle extract was centrifuged in a Spinco preparative ultracentrifuge at 100,000 G for eight hours at 4° C. The radioiron incorporation into heme was studied in uncentrifuged extract, supernatant solution and residue. Since myoglobin remains in the supernatant under these conditions, none was present in the samples of residue. Therefore, activity was determined by the isolation of heme instead of myoglobin. The data presented in Table II show that supernatant solution had much less activity than residue, which had essentially the same actvity as the initial extract.

Effects of dialysis and of sulfhydryl compounds

It has been shown that sulfhydryl groups are required for optimal activity in an avian hemolysate system for the synthesis of heme from protoporphyrin and iron (13, 14). For this reason, the effects of dialysis and of sulfhydryl compounds were studied in the present system.

Dialysis against distilled water, 4° C., for 18 hours, resulted in almost complete loss of activity. The addition of cysteine to dialyzed extracts resulted in partial restoration of activity. In undialyzed extracts, cysteine addition resulted in a twofold increase in radioiron uptake. Similar results were obtained when reduced glutathione was used instead of cysteine. These data are shown in Table III.

Effects of inhibitors

The effect of *p*-chloromercuriphenyl sulfonic acid, a sulfhydryl inhibitor, was studied. This compound, at a concentration of 2.5×10^{-3} M, resulted in 69 per cent inhibition of radioiron incorporation into the myoglobin. At a concentration of 2.5×10^{-2} M, the inhibition was 97 per cent. Incubation in the presence of 1×10^{-3} M potassium cyanide resulted in complete inhibition of radioiron incorporation.

Effect of glycine or δ-aminolevulinic acid

The effect of glycine and δ-aminolevulinic acid on radioiron uptake was measured in the supernatant solution of a muscle homogenate. In addition, the effect of glycine alone and with succinate was studied in muscle brei. In three experiments with muscle extract, augmentation could not be demonstrated with concentrations of glycine varying from 10×10^{-2} to 5×10^{-3} M. The addition of 1×10^{-4} M δ-aminolevulinic acid led to a two- to fourfold increase in radioiron uptake in two out of three experiments (Table IV). Using the same Tris concentration for brei as was used in supernatant extracts, the pH of the mixtures dropped to 6.4 during incubation, and no radioiron uptake occurred. By using 1.0 M Tris and incubating brei immediately after grinding the muscle, the pH could be controlled. With this technique, glycine alone increased radioiron incorporation, while glycine plus succinate gave further augmentation (Table V).

Human material

The incorporation of radioiron into myoglobin from extracts of human muscle obtained from two nondystrophic and two dystrophic subjects was studied. The autopsies were performed 12 to 20 hours after death. The data are shown in Table VI.

In the muscle extracts from all four subjects, the addition of protoporphyrin resulted in a two-

TABLE III

The effect of cysteine or reduced glutathione on uptake of radioiron

	Undialyzed extract	Dialyzed extract
	cpm/μg. Fe	*cpm/μg. Fe*
Cysteine concentration		
0	147	15
1×10^{-3} M		87
5×10^{-3} M		127
1×10^{-2} M	275	177
Glutathione concentration		
0	33	
1×10^{-2} M	70	

TABLE IV

Effect of glycine and δ-aminolevulinic acid on radioiron uptake in dog muscle extract

Experiment	Substrate	Concentration	Control	Substrate added
			cpm/μg. Fe	*cpm/μg. Fe*
1	Glycine	5×10^{-3} M	10	9
2	Glycine	5×10^{-2} M	3	4
3	Glycine	10×10^{-2} M	3	2
4	δ-Aminolevulinic acid	1×10^{-4} M	12	53
5	δ-Aminolevulinic acid	1×10^{-4} M	2.5	2.5
6	δ-Aminolevulinic acid	1×10^{-4} M	30	68

fold increase in the incorporation of radioiron into myoglobin. In the muscle extracts from the one nondystrophic subject to which cysteine was added, a further twofold increase in iron uptake was observed.

DISCUSSION

It may be concluded fom these studies that the supernatant solution from a dog muscle homogenate is capable of radioiron incorporation into myoglobin heme. The uptake of radioiron was markedly enhanced by the addition of protoporphyrin and was moderately increased in the presence of δ-aminolevulinic acid. Glycine plus succinate gave augmentation in muscle brei. In these respects, the results obtained with muscle extracts closely resemble those found with avian erythrocytes (14,15). In other ways, as well, the similarity between muscle extracts and other tissues is striking. The optimal protoporphyrin concentration, the pH of optimal activity, the augmentation by cysteine, and the inhibition by sulfhydryl inhibitors all parallel results obtained with nucleated erythrocytes (14,15) and liver (4). These findings are in keeping with the hypothesis that the mechanism for heme synthesis, which has been best demonstrated in avian erythrocytes, is a general mechanism (16), with only minor differences from tissue to tissue.

TABLE V

Effect of glycine and succinate on radioiron uptake in dog muscle brei

	cpm/μg. Fe
Brei control	7
Brei + protoporphyrin	59
Brei + glycine, 5×10^{-2} M	39
Brei + glycine, 5×10^{-2} M and succinate, 5×10^{-2} M	57

Since the majority of the present studies were carried out at the step in myoglobin synthesis involving the incorporation of iron into heme, certain comments are in order concerning the evidence that the process demonstrated in muscle extracts is an enzymic one. It has been stated that, under conditions in which protoporphyrin is not in a colloidal state, chemical, nonenzymic insertion of iron can occur to form heme (17,18). Several observations suggest that this was not the case in the present studies. First, heat and minimal concentration of potassium cyanide and parachloromercuriphenylsulfonic acid inhibited radioiron incorporation. Second, the supernatant solution obtained after ultracentrifugation, which contained amounts of myoglobin, protoporphyrin and radioiron like those present in whole extract, was much less active than either whole extract or residue. It should be noted also that controls consisting only of iron and protoporphyrin yielded practically no heme under the conditions of these experiments (Table II). Finally, the finding of

TABLE VI

Radioiron incorporation in myoglobin of human subjects

Diagnosis	Control	With protoporphyrin 1×10^{-5} M	With protoporphyrin and cysteine 1×10^{-2} M
	(*cpm/μg. Fe*)	(*cpm/μg. Fe*)	(*cpm/μg. Fe*)
Nondystrophic E. T.	4	10	
Nondystrophic E. H.	10	25	50
Myotonic dystrophy A. L.	7	16	
Myotonic dystrophy R. H.	1	5	

optimal activity at a pH near neutrality with lower activity on each side of this optimum is in accord with the conclusion that the process is an enzymic one.

With the exception of one extract from nondystrophic muscle, only low levels of radioactivity were obtained with human muscle. The autolysis which had occurred in the long periods that had elapsed between death and study of radioiron uptake may have been responsible for the low levels of uptake. In each instance, however, protoporphyrin increased the incorporation of radioiron into myoglobin. In addition, cysteine was found to augment this process further, in one instance. It seems reasonable to conclude, therefore, that radioiron incorporation into myoglobin heme occurred in muscle extracts from all these patients. Within these limitations, no differences were observed between dystrophic and nondystrophic muscle.

SUMMARY

1. Radioiron was incorporated into the heme of myoglobin in the supernatant solutions from dog muscle homogenates.

2. Protoporphyrin and δ-aminolevulinic acid augmented the incorporation of radioiron. Cysteine and reduced glutathione were also found to increase radioiron uptake. Glycine plus succinate augmented radioiron uptake in muscle brei.

3. Heat, potassium cyanide and parachloromercuriphenylsulfonic acid inhibited this process.

4. The conditions which favor iron incorporation into myoglobin heme closely resemble those known to favor heme biosynthesis in other tissues.

5. Preliminary observations suggest that extracts from both normal and dystrophic human muscle are capable of carrying out this process.

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IRON ENZYMES IN IRON DEFICIENCY. VI. ACONITASE ACTIVITY AND CITRATE METABOLISM

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Iron serves vital functions in the economy of the body in a variety of ways. Its role as an integral part of biologically important substances is well known. In heme, it forms a part of the oxygen-carrying compounds, hemoglobin and myoglobin, and of the cytochromes, cytochrome oxidase, catalase and perioxidase. Another enzyme, succinic dehydrogenase, appears to contain iron (1), but not bound as heme. Physiologic vasodepressor activity has also been ascribed to the iron storage compound, ferritin (2). Less well known, perhaps, is the relatively recently described function of iron as a cofactor in several important enzymatic reactions (3–5). Knowledge of this function of iron served as the basis for the current investigation.

In previous studies of the pathologic physiology of the iron-deficiency state, we have examined the fate of the heme compounds: hemoglobin, cytochrome c (6, 7), catalase (7, 8), cytochrome oxidase (9), and succinic dehydrogenase (10). Contrary to the earlier conclusions of Hahn and Whipple (11), but in agreement with those of Gubler, Cartwright and Wintrobe (12), we found that varying degrees of depletion in several iron enzymes occurred in the iron-deficiency state. In the current study, we have undertaken measurement of aconitase, an enzyme that is not known to contain iron, but that appears to require iron as a specific cofactor (5). Dialyzed solutions of aconitase lose their activity. Dickman and Cloutier (5) demonstrated that the addition of iron and a reducing agent reactivated the enzyme. None of the many other ions tested had the capacity to reactivate aconitase (5, 13).

Aconitase catalyzes the biologically important equilibrium between citrate, isocitrate and cis-aconitase. Takeda and Hara (14) have reported that activity of aconitase is diminished in livers of guinea pigs poisoned with α-α'-dipyridyl, and that marked citric acid diuresis occurred in these animals, especially after loading with citrate and butyrate. While we cannot accept Takeda's implication that α-α'-dipyridyl-poisoned animals are iron-deficient in a sense in any way comparable to iron deficiency produced by bleeding or by a low iron diet, it seemed worthwhile to explore aconitase activity and the metabolism of exogenously administered citrate in truly iron-deficient animals and humans.

MATERIALS AND METHODS

Unless otherwise indicated, female Holtzman strain rats have been used throughout these experiments. All animals were fed the iron-poor diet that we have described previously (7), and were given water rendered iron free by passing it over an ion exchange resin. Unless otherwise indicated, each control rat was given 25 mg. of iron, as iron-dextran (Imferon®),[1] intramuscularly into the hamstring muscles in two divided doses, one at the beginning of the experiment, the other, two weeks later. The animals were not fasted prior to sacrifice. Each animal was exsanguinated from the heart under ether anesthesia. All of the organs that were removed were placed into chilled vessels.

The method of aconitase assay was based on that described by Anfinsen (15). Tissues were homogenized in ice cold 0.2 M phosphate buffer, pH 7.40, with a Potter-Elvejhem type homogenizer, and the diluted homogenate was filtered through Schleicher and Schuell No. 604 filter paper. Filtrates were kept at 0° C. and assays were carried out within an hour. Unless otherwise stated, 0.1 and 0.2 ml. aliquots of filtered tissue homogenate were added to 4.4 or 4.3 ml. of 0.2 M phosphate buffer, pH 7.4, in a 37° C. water bath. The reaction was started by the addition of substrate, 0.5 ml. of cis-aconitate solution. The substrate was freshly prepared for each assay by allowing an aqueous solution of cis-aconitic acid anhydride (Sigma Chemical Corporation) to remain at room temperature for one hour, then neutralizing with 0.1 ml. 2 N NaOH per 2.76 mg. anhydride. The volume was then adjusted with buffer to give a concentration of 15.6 mg.

[1] Supplied through the courtesy of Lakeside Laboratories, Milwaukee, Wisconsin.

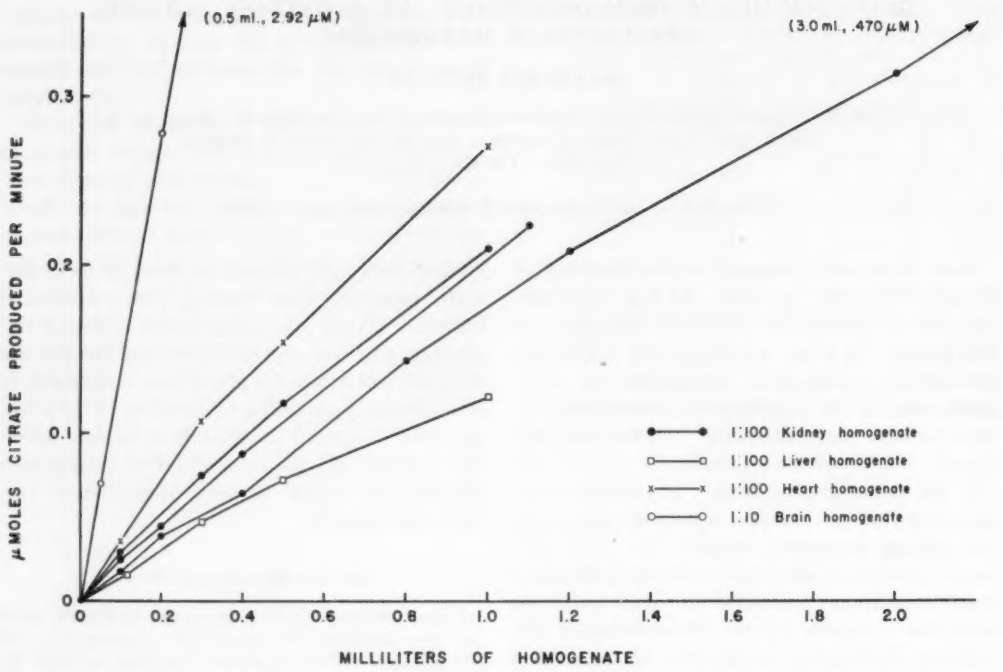

FIG. 1. THE RELATIONSHIP BETWEEN THE AMOUNT OF CITRATE PRODUCED FROM CIS-ACONITATE AND THE VOLUME OF HOMOGENATE ADDED

Assay system: cis-aconitate, 7.8 mg. (as anhydride); tissue homogenate, as indicated; final volume 5 ml. in 0.2 M phosphate buffer, pH 7.4; temperature 37° C.

(as anhydride) per ml. Fifteen minutes after addition of substrate, the reaction was stopped. In the case of heart, kidney and liver, this was done by adding 1 ml. of the reaction mixture to 0.5 ml. of 50 per cent (V/V) sulfuric acid in a tube marked at the 10 ml. mark. The total volume was then brought to approximately 5 ml. with distilled water. In the case of brain homogenate, the reaction was stopped by adding 5 ml. of 20 per cent trichloracetic acid to the entire 5 ml. reaction mixture; citric acid determinations were done on 5 ml. of the filtrate. Citric acid determinations were carried out according to our modification of the pentabromacetone method (16). In this assay system, the quantity of citric acid produced is approximately linear with respect to time of reaction and to volume of tissue extract added (Figure 1). One unit of enzyme activity has been defined arbitrarily as that amount of enzyme that will, in the assay system described, convert 10^{-7} Moles of cis-aconitic acid to citric acid per minute.[2] Because of the slight departure from linearity that was sometimes observed when the amount of citric acid formed was compared with the amount of tissue extract added (Figure 1), two dilutions of tissue were assayed. These dilutions were chosen so that one sample usually contained less than one unit and the other contained more than one unit of enzyme activity. The average of these two determinations has been reported. One-tenth and 0.2 ml. of the following tissue dilutions were found to be appropriate: kidney, 1 to 25 or 1 to 40; heart, 1 to 50; brain, 1 to 20; liver, 1 to 20.

The method used for assaying the aconitase activity of human white cells has been described in detail elsewhere (17).

[2] In two experiments, an assay system containing 3.9 mg. cis-aconitic acid as an anhydride was used. It had been determined empirically that one unit of enzyme in this system converts 0.85×10^{-7} Moles of cis-aconitic acid to citrate per minute.

EXPERIMENTAL AND RESULTS

A. The reactivation of aconitase by iron

A one to 800 homogenate was prepared from the kidney of a normal rat in 0.2 M phosphate buffer, pH 7.4 The aconitase activity of the homogenate was assayed immediately and at intervals after incubation at 37° C. Solutions containing 0.03 M ferrous sulfate, 0.03 M ascorbic

acid, and both were prepared and their pH adjusted to 7.4. After one hour, one volume of one of each of these solutions was added to each of three nine-volume aliquots of the incubating mixture. As a control, one volume of buffer was added to a fourth aliquot. Aconitase activity was determined at intervals. The results of this experiment, showing the time course of partial reactivation of aconitase by iron and ascorbate, are shown in Figure 2. No reactivation was achieved by the addition of either iron or ascorbate alone.

The effect of different concentrations of iron and ascorbic acid has been investigated in a similar fashion. In three experiments, one to 800 kidney homogenate, 75 to 80 per cent inactivated by incubating for one hour at 37° C., was reactivated for 10 minutes with iron and ascorbate. Aconitate was then added and enzyme activity measured in the usual manner. It is apparent that complete reactivation of incubated enzyme could be achieved regularly when sufficiently high concentrations of iron and ascorbic acid were used. The results of these studies are given in Figure 3.

B. Aconitase activity of organs of normal and iron-deficient rats

Four 23-day-old rats were sacrificed and the hemoglobin, hematocrit and plasma iron values and aconitase activity of kidneys, hearts and livers were determined. Eighteen 23-day-old rats were given an iron-poor diet, nine of these being injected with iron-dextran. After 10 days, four iron-deficient and four iron-treated (control) rats were sacrificed, and after 26 days, the remaining five iron-deficient and five iron-treated (control) animals were sacrified. The hemoglobin, hematocrit and plasma iron values and organ aconitase assays of these animals are presented in Table I. A well-marked decrease in aconitase activity of the kidneys of the iron-deficient animals, compared with that of the control animals, was found. In sharp contrast, the aconitase activity of the other organs studied was normal, with the possible exception of a slight reduction of heart enzymatic activity. The enzymatic activity of mixtures of equal volumes of homogenates from iron-deficient and normal animals was the arithmetic mean of the two: no inhibition of normal kidney enzyme or activation of iron-deficient kidney enzyme could be demonstrated. Rats that had always been fed stock diet had kidney aconitase activity comparable with that of the iron-dextran controls. Administration of iron-dextran to such rats did not cause an elevation of kidney aconitase activity.

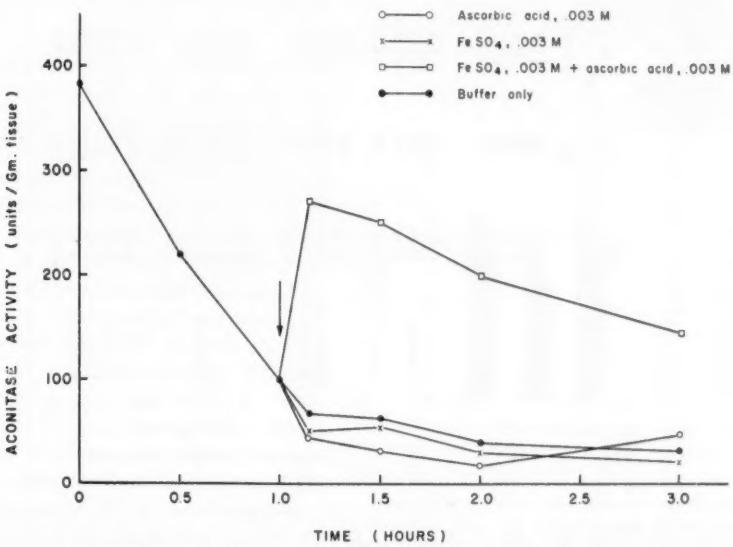

FIG. 2. THE REACTIVATION OF ACONITASE BY IRON AND ASCORBIC ACID

TABLE I

Aconitase activity of organs from iron-deficient and control rats

Age	Duration of iron treatment	Group	Rat No.	Wt.	Hb	Hematocrit	Plasma iron	Kidney		Heart		Liver		Brain	
								Aconitase	Wt.	Aconitase	Wt.	Aconitase	Wt.	Aconitase	Wt.
days	*days*			*Gm.*	*Gm. %*	*%*	*μg./100 ml.*	*units/Gm.*	*Gm.*	*units/Gm.*	*Gm.*	*units/Gm.*	*Gm.*	*units/Gm.*	*Gm.*
23	0		1–A	60	11.3	38	320	141	0.71	262	0.30	119	2.58		
			2–A	58	10.5	34	110	162	0.63	305	0.26	114	2.07		
			3–A	51	10.2	34	210	118	0.63	224	0.24	107	2.08		
			4–A	52	11.0	34	180	190	0.54	280	0.19	125	1.91		
35	10	Control	1–B	94	11.9	40	100	345	0.81	492	0.31	196	4.15	84	1.30
			2–B	79	11.6	38	60	369	0.64	521	0.26	188	2.94	103	1.20
			3–B	91	11.8	39	170	342	0.77	502	0.30	152	3.46	106	1.31
			4–B	85	12.7	40	88	355	0.71	510	0.30	175	3.24	118	1.25
	0	Iron-deficient	5–B	85	9.9	34	10	202	0.78	414	0.34	164	3.32	93	1.25
			6–B	96	11.2	37	64	236	0.74	421	0.33	158	3.44	75	1.29
			7–B	94	8.0	27	7	158	0.74	404	0.36	137	3.42	105	1.33
			8–B	97	11.1	36	50	232	0.90	531	0.34	152	3.64	108	1.35
49	24	Control	1–C	119	12.7	40	235	369	0.89	415	0.44	141	5.26	75	1.42
			2–C	121	12.3	39	265	309	1.14	390	0.49	164	5.44	82	1.36
			3–C	119	13.1	41	107	347	0.97	418	0.45	154	5.12	93	1.42
			4–C	126	13.1	39	230	415	1.22						
			5–C	115			190	318	1.00						
	0	Iron-deficient	6–C	119	7.9	26	5	142	0.94	336	0.55	163	4.65	94	1.41
			7–C	122	10.2	33	50	222	1.02	399	0.48	156	4.68	100	1.22
			8–C	127	9.2	30	7	205	1.04	316	0.51	131	5.56	85	1.35
			9–C	110	9.0	33	14	202	0.81						
			10–C	121	10.4	31	7	199	0.99						

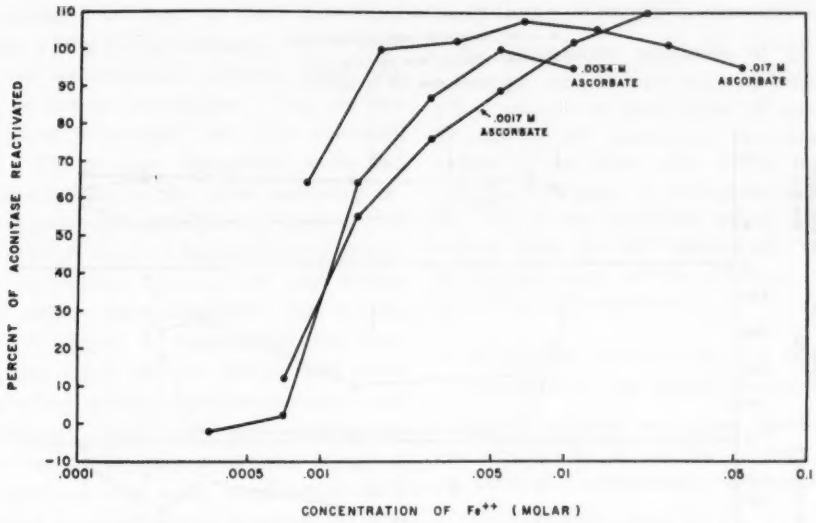

FIG. 3. THE REACTIVATION OF ACONITASE BY VARIOUS CONCENTRATIONS OF IRON AND ASCORBATE

In each of the three experiments, normal rat kidney homogenate diluted one to 800 was incubated for one hour at 37° C. The concentrations of iron and ascorbate indicated were added and an equal volume of buffer was added to a control tube. Aconitase activity was determined after an additional 10 minutes' incubation. Seventy-five to 80 per cent of the enzyme was inactivated in the control tube. The effect of iron and ascorbate is expressed as the percentage of inactivated enzyme which was reactivated.

C. An attempt to reactivate aconitase of kidneys from iron-deficient rats with iron and ascorbic acid

Tissue homogenates from iron-deficient and control rats were incubated with ascorbate and ferrous sulfate, both in final concentration of 0.003 M, for one-half hour. The aconitase activity of these homogenates was compared with that of control homogenates that had not been incubated with iron and ascorbate acid. No "reactivation" of enzyme in homogenates prepared from iron-deficient animals could be demonstrated (Figure 4). The use of ferrous sulfate and ascorbate in a higher concentration, 0.005 M, also resulted in no "reactivation." As indicated under *B* above, normal kidney homogenate also failed to "activate" iron-deficient kidney homogenate. Similarly, 3 ml. of one to 400 normal kidney homogenate completely inactivated by heating to 100° C. for five minutes or to 60° C. for 30 minutes, or partially inactivated by incubating at 37° C. for two hours, failed to "reactivate" iron-deficient kidney homogenate. In point of fact, approximately 33 per cent inhibition of the enzymatic activity of kidney homogenate from iron-deficient rats was observed.

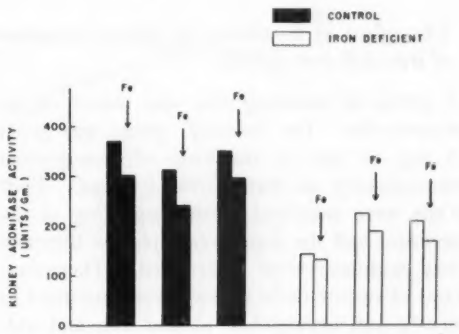

FIG. 4. THE FAILURE OF IRON AND ASCORBATE TO "REACTIVATE" ACONITASE FROM NORMAL AND IRON-DEFICIENT RAT KIDNEYS

Bars under arrows represent aconitase activity of homogenates that had been incubated for one-half hour with ascorbate and ferrous sulfate, both in final concentration of 0.003 M.

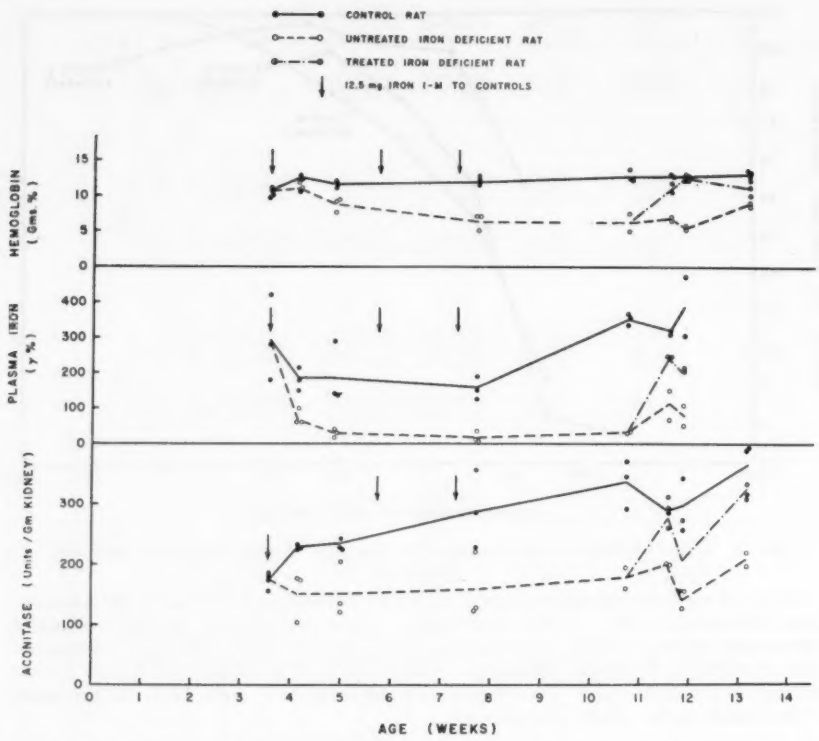

FIG. 5. THE EFFECT OF INTRAGASTRIC ADMINISTRATION OF IRON ON THE HEMOGLOBIN AND PLASMA IRON VALUES, AND ON KIDNEY ACONITASE ACTIVITY OF IRON-DEFICIENT RATS

Each point represents the value obtained from a single rat. The lines connect the means of each group. Intragastric iron therapy, 2 mg. Fe^{++} per day, was initiated when the rats were 76 days old.

D. The effect of treatment on kidney aconitase of iron-deficient animals

A group of weanling rats was placed on an iron-poor diet. The "control" group was given 37.5 mg. of iron in the form of iron-dextran intramuscularly in three divided doses. Four animals were sacrificed at the beginning of the experiment and the hemoglobin, plasma iron and kidney aconitase were determined. Thereafter, groups of two or three animals were sacrificed at intervals, and hemoglobin, plasma iron and kidney aconitase assays were carried out. Approximately seven weeks after initiation of the experiment, one-half of the iron-deficient group was treated by the daily intragastric administration of 2 mg. of iron in the form of iron sulfate in 0.01 N hydrochloric acid. A prompt return of kidney aconitase activity toward normal values was observed in the iron-treated group. The individual values were too scattered to warrant conclusions about the rate of return of aconitase to normal activity, as compared with hemoglobin and plasma iron values (Figure 5).

E. The effect of anemia on aconitase activity of non-iron-deficient animals

Holtzman strain, retired male breeder rats, weighing 450 to 510 Gm., were placed on the iron-poor diet and were divided into four groups. A control group of four rats received no treatment. Another group of four animals was injected subcutaneously with 10 or 20 mg. of phenylhydrazine at two to four day intervals (Figure 6). The other two groups of six rats

each were bled 5 ml. from the heart three times weekly for a total of six bleedings. Six of these animals were intramuscularly injected weekly with 12.5 mg. of iron as iron-dextran. Only six bled animals, three iron-treated and three untreated, survived. The average hemoglobin levels and treatment schedules of the entire surviving experimental group are presented in Figure 6. At the end of the 27 days, all animals were sacrificed and the hemoglobin, plasma iron and kidney aconitase activity were measured. The results, presented in Figure 7, demonstrate that iron-deficient rats (bled, but not treated with iron) had diminished kidney aconitase activity, even after relatively short-term iron deprivation. Anemia, induced by phenylhydrazine injections or by bleeding when iron supplements were given, failed to affect kidney aconitase activity.

F. Aconitase activity of human white cells

Semiquantitative estimation of the aconitase activity of human white cells, reported elsewhere (16), revealed a mean value of approximately 98 units per 10^{10} leukocytes. Aconitase determinations of the white cells of four iron-deficient subjects have given an average aconitase value of 88. All of the individual values (106, 94, 77 and 77 units per 10^{10} leukocytes) were within the normal range, and the difference in means is not statistically significant.

G. Metabolism and excretion of a citrate load in iron-deficient and normal subjects

Fasting subjects were given disodium hydrogen citrate, 130 mg. per Kg. of body weight, in the form of a commercial preparation.[3] Citrate

[3] Citralka®, Parke, Davis and Co.

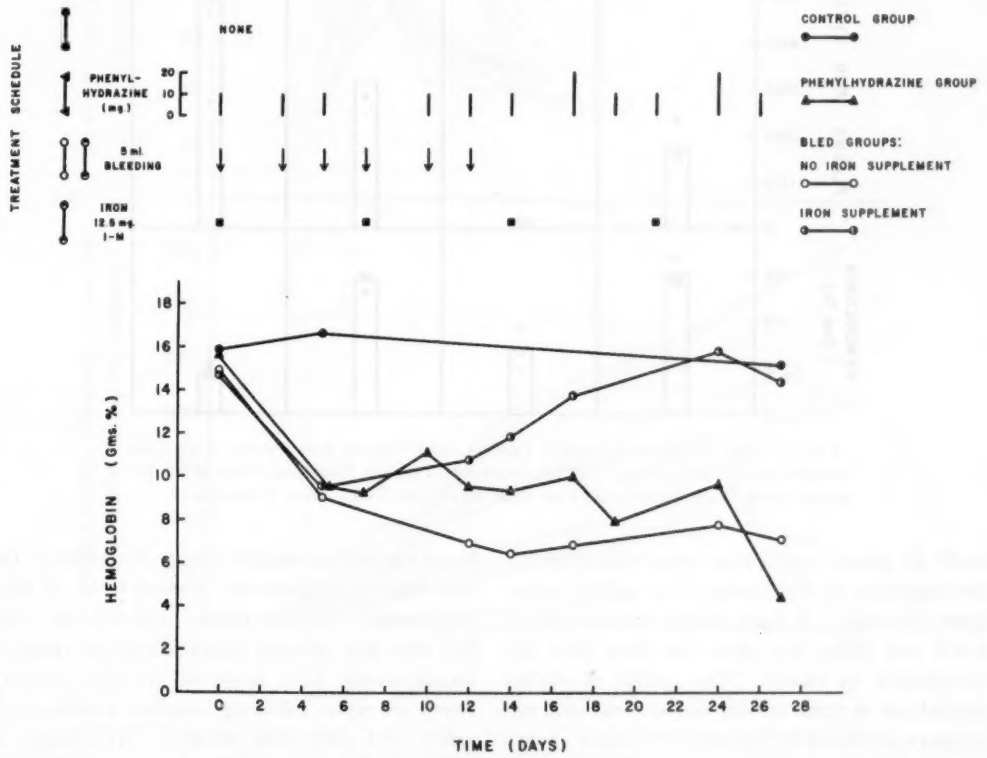

FIG. 6. TREATMENT SCHEDULES AND AVERAGE HEMOGLOBIN LEVELS OF CONTROL RATS, PHENYLHYDRAZINE-TREATED RATS, BLED RATS INJECTED WITH IRON-DEXTRAN AND BLED RATS WITHOUT IRON SUPPLEMENTS

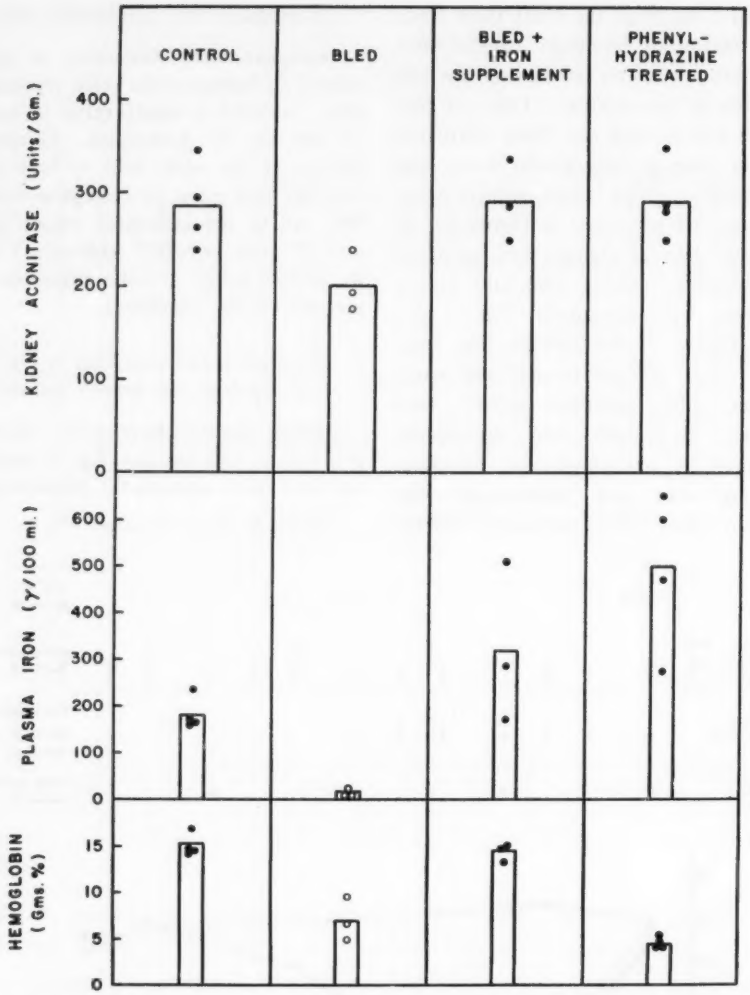

FIG. 7. THE HEMOGLOBIN LEVELS, PLASMA IRON VALUES AND KIDNEY ACONITASE ACTIVITY OF CONTROL RATS, PHENYLHYDRAZINE-TREATED RATS AND BLED RATS INJECTED WITH IRON-DEXTRAN AND OF BLED RATS NOT GIVEN IRON SUPPLEMENT

levels in plasma and urine were measured before ingestion of the loading dose and at subsequent intervals. A light breakfast consisting of a roll and coffee was given one hour after administration of citrate. The results of studies carried out in three normal and six iron-deficient subjects are shown in Figure 8. Pertinent clinical and hematologic data are presented in Table II. The slightly higher plasma average levels and lower cumulative urinary citrate excretion of the iron-deficient subjects are deemed to be of little importance. In one mildly iron-deficient subject with high urinary citrate excretion, repeated measurements were made of six hour urinary citrate excretion following standard loading, both before and after iron therapy. No change in citrate excretion could be demonstrated (Figure 9).

DISCUSSION

A decrease in aconitase activity has been demonstrated in the kidneys of iron-deficient rats. Weanling rats deprived of iron failed to develop the rapid increase in kidney aconitase activity that occurs when iron is administered to young rats. The activity of this enzyme decreased in mature animals rendered iron-deficient by repeated bleeding while on an iron-poor diet. It is of particular interest that the decrease in enzymatic activity in kidneys from iron-deficient rats did not appear to be due merely to a lack of iron acting as a cofactor for aconitase activity, since addition of iron failed to "reactivate" aconitase from iron-deficient animals. Normal kidney tissue also failed to reactivate enzyme from iron-deficient kidneys, and it may therefore be presumed that the decrease of aconitase activity was due to actual depletion of the protein moiety of the enzyme. In this respect, aconitase may be regarded to be similar to apoferritin, which is synthesized in response to iron (18), and to the

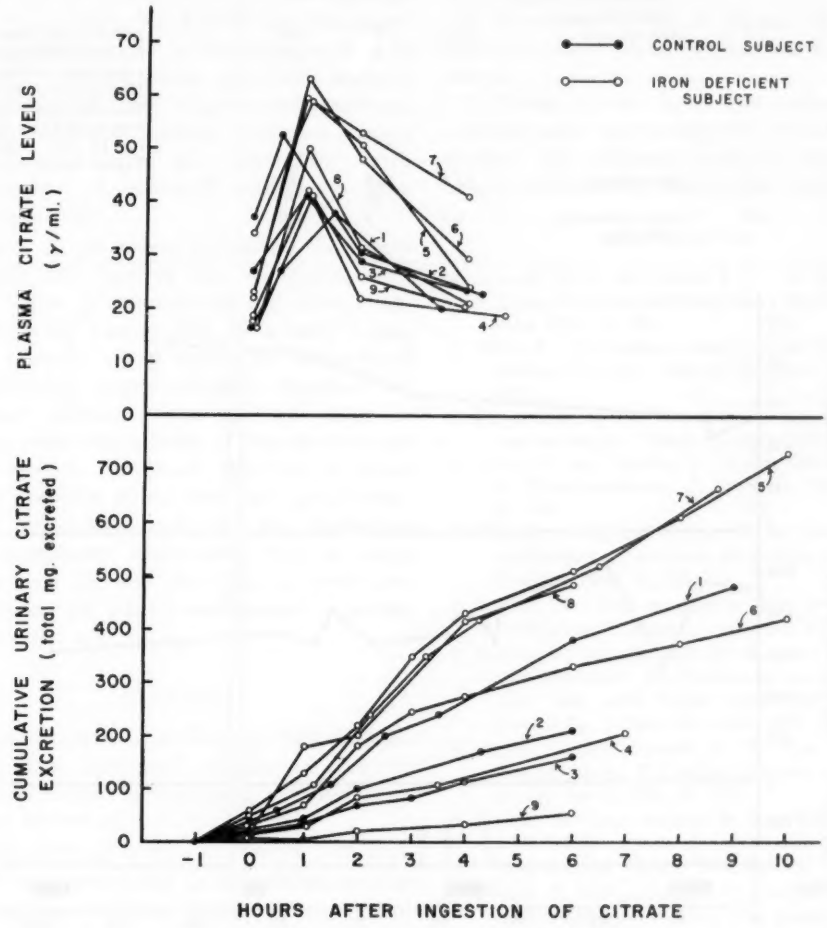
CONTROL SUBJECT
IRON DEFICIENT SUBJECT
PLASMA CITRATE LEVELS (γ/ml.)
CUMULATIVE URINARY CITRATE EXCRETION (total mg. excreted)
HOURS AFTER INGESTION OF CITRATE

FIG. 8. PLASMA CITRATE AND CUMULATIVE URINARY CITRATE EXCRETION IN THREE NORMAL AND SIX IRON-DEFICIENT SUBJECTS

At time = 0, 130 mg. of disodium hydrogen citrate was given per Kg. of body weight. Pertinent clinical and hematological data for each patient are given in Table II.

TABLE II

Clinical and laboratory findings of nine subjects studied after citrate loading

Patient No.	Sex	Age	Clinical diagnosis	Hb	Plasma iron	Serum unsaturated iron-binding capacity	Marrow iron	Remarks
				Gm. %	*μg./100 ml.*	*μg./100 ml.*		
1	F	28	Cardiac arrhythmia	13.3			++	
2	M	22	Normal male					
3	M	25	Normal male					
4	F	21	Pseudohemophilia (no recent pathological bleeding)	10.9	5	540		
5	F	22	Menorrhagia	4.8	5	450		
6	F	25	Menorrhagia (inactive rheumatic heart disease)	10.8	5	540		
7	F	35	Menorrhagia	12.7	50 50	140 160		Patient had been treated for iron deficiency a year previously and her normal Hb was known to be higher than the present level. Hb rose to 14.5 Gm. % following IV iron therapy (see Figure 9).
8	F	43	Menorrhagia	13.6	46 44	180 270	0	
9	F	62	Gastrointestinal bleeding	6.7	5	400		

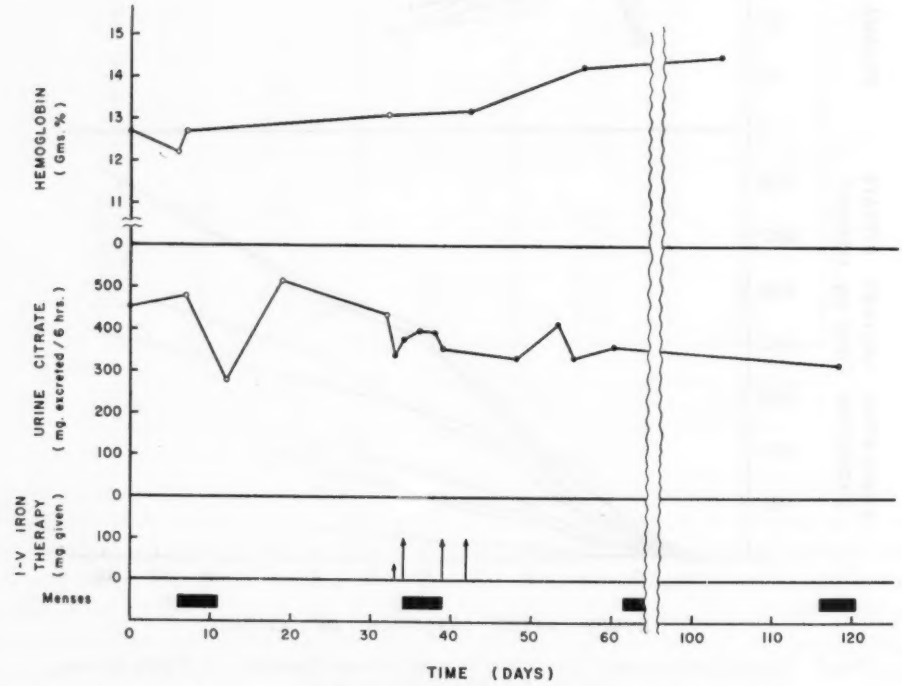

FIG 9. CUMULATIVE SIX HOUR URINARY CITRATE EXCRETION OF SUBJECT NO. 7

Measurements of citrate excretion were made following oral citrate loading on repeated occasions, before and after intravenous administration of iron in the form of saccharated iron oxide.

globin moiety of hemoglobin, synthesis of which appears to be inhibited in iron deficiency (19).

Takeda and Hara (14) have reported that poisoning with α-α'-dipyridyl results in greatly increased urinary excretion of citric acid by guinea pigs, especially after they had been fed large quantities of citrate and butyrate. Using rats as the test animal, we have been unable to duplicate these results, using our modification of the pentabroacetone method for citrate analysis (16). The less specific sodium sulfide (20) method used by Takeda and Hara gives a false positive reaction for citric acid with α-α'-dipyridyl (21). It is possible that Takeda and Hara measured α-α'-dipyridyl, or a metabolic product as citric acid. However, even true citric aciduria, if it occurs under these experimental conditions, does not necessarily indicate profound changes in intracellular citric acid metabolism: citric acid excretion is extremely sensitive to urine pH changes (22).

In spite of our failure to confirm the results of Takeda and Hara in rats, we studied citric acid excretion in iron-deficient and normal subjects following loading with citric acid, hoping that a clinically useful means for diagnosis of iron deficiency might emerge. However, no significant difference was observed either in blood or urine citrate levels of iron-deficient and normal subjects. Obviously, this does not prove that the operation of the citric acid cycle is normal in iron-deficient subjects: the metabolism of an exogenously administered dose of citrate may be quite different from that of citrate produced within the cell by condensation of acetate and oxalacetic acid.

SUMMARY

1. The observation of Dickman and Cloutier (5, 13), that inactivated solutions of aconitase may be reactivated by iron and a reducing agent, has been confirmed.

2. The aconitase activity of kidneys from iron-deficient rats was found to be reduced consistently, but the aconitase activity of the hearts, livers and brains of iron-deficient rats was normal.

3. Aconitase from the kidneys of iron-deficient rats could not be "reactivated" by the addition of iron and ascorbate, homogenate of normal rat kidney, or heat-inactivated normal rat kidney homogenate. This finding is interpreted as indicating an actual depletion of the protein moiety of aconitase in iron-deficient animals.

4. Treatment of iron-deficient rats with iron restored their kidney aconitase activity to normal.

5. Anemia, induced by the administration of phenylhydrazine or by repeated bleeding of rats given supplemental iron, failed to reduce kidney aconitase activity; blood loss anemia in rats not given iron supplement resulted in a decrease in kidney aconitase activity.

6. Aconitase activity of human white blood cells from iron-deficient subjects was found to be normal.

7. Plasma citrate levels and urinary citrate excretion after oral loading with citrate has been studied. No difference could be demonstrated between normal and iron-deficient subjects.

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UREA METABOLISM IN MAN*

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Urea has long been considered to be metabolically inert. However, recent studies with isotopic urea in several species of animals have revealed that urea is hydrolyzed in the alimentary tract even when administered parenterally (1–5). This breakdown is presumably due to the action of bacterial urease, since urea metabolism can be eliminated or reduced to negligible proportions by the administration of antibiotics.

In the studies reported herein, labeled urea was administered intravenously to normal subjects and the rate of extrarenal disposal was determined. Both N^{15}- and C^{14}-labeled materials were employed in order to minimize the problem of reincorporation of tracer atoms into urea. In the case of N^{15}, enriched ammonia produced by urea hydrolysis in the gut becomes reincorporated into newly formed urea. In the case of C^{14}, recent evidence (6) suggests that reincorporation into urea of labeled CO_2, formed by bacterial ureolysis in the gut, may also occur; a conclusion supported by the present experiments.

Despite these limitations, the results indicate that 15 to 30 per cent of the urea being synthesized in normal subjects is continually being destroyed. This breakdown can be eliminated by oral administration of neomycin.

METHODS

Urea containing 60.9 atoms per cent N^{15} was synthesized by the ammonolysis of diphenyl carbonate (7) and purified by recrystallization.[1] The resulting product had a melting point of 130° C. (uncorrected). The nitrogen content was 46.7 per cent (calculated 46.7 per cent).

C^{14}-labeled urea containing 1 mc. of C^{14} per mMole was obtained from Volk Laboratories under authorization by the Atomic Energy Commission. Both preparations were prepared for use by dissolving in sterile saline solution. Tests for pyrogenicity and bacterial contamination were negative.

The subjects were normal healthy volunteers who continued their usual meals and activities during the studies. One glass of water per hour was ingested for four to eight hours following administration of labeled urea. Urinary catheters were not employed.

Measured portions of labeled urea (25 to 100 mg. of N^{15}-urea or 2.5 μc. of C^{14}-urea) were injected intravenously. At intervals, venous blood samples were obtained, and immediately thereafter, the subjects voided. In the N^{15} experiments, the urine samples were acidified immediately to prevent loss of ammonia.

The nitrogen content and atoms per cent excess N^{15} were determined in urinary ammonia, urea and total nitrogen and in serum urea. Urine ammonia was isolated on permutit which was then washed five times with distilled water. Ammonia-free urine and a tungstic acid filtrate of serum were treated with urease according to the method of Van Slyke and Cullen (8). A sample of urine was also subjected to Kjeldahl digestion overnight. After the addition of alkali, ammonia from these various fractions was steam distilled into boric acid solution and titrated with 0.1 N HCl. The recovery of urea was determined simultaneously in each experiment and averaged 98 per cent. The solutions remaining after titration were evaporated to a small volume and allowed to react with sodium hypobromite in Rittenberg tubes. The atoms per cent N^{15} in the evolved nitrogen gas was determined in the mass spectrometer (Consolidated model 21–201). Atoms per cent excess N^{15} was determined by subtracting values for atoms per cent N^{15} in control samples of blood and urine. Compared to an ammonium chloride standard, the control samples contained a biological excess of N^{15} of less than 0.004 atoms per cent. The maximum error of the N^{15} analyses was ± 0.002 atoms per cent.

In the C^{14} experiments, total urea was determined by the method of Conway (9). C^{14}-urea was determined as follows: aliquots of blood or urine were acidified with HCl and shaken with a few drops of caprylic alcohol in order to remove all bicarbonate. Sufficient alkali was added to restore the pH to approximately 7, and aliquots were placed in 50 ml. flasks containing a center well. One ml. of 3 per cent urease solution (Sigma Chemical Co.) and 1 ml. of phosphate buffer, pH 7.2, were added and the flasks were stoppered. After 20 minutes shaking at 37° C., dilute H_2SO_4 was injected through the stopper. Shaking was

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* An abstract of this work has been published (Urea breakdown in normal man. J. clin. Invest. 1957, **36**, 933). The opinions presented herein are those of the authors and do not necessarily reflect the views of the United States Navy.

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[1] Dr. W. J. McCarville kindly carried out this synthesis.

continued for another hour. In the earlier experiments, the center well contained 25 per cent NaOH, CO_2-free, which was transferred to centrifuge tubes, mixed with 10 ml. 1 per cent $BaCl_2$ in CO_2-free water, and centrifuged. The precipitated barium carbonate was washed with CO_2-free 50 per cent ethanol, suspended in 95 per cent ethanol and transferred to weighed planchets. The planchets were dried, reweighed and counted in a flow counter for a total of 10,000 counts. After correction for background and self-absorption, the results were expressed in terms of an aliquot of the injected solution analyzed simultaneously.

In the later experiments, the center well contained 0.2 ml. of Hyamine®, 0.5 N. This was transferred with the aid of 1.5 ml. toluene to specimen vials, mixed with 15 ml. toluene containing diphenyl oxazole, 1 N, capped, cooled and counted in a Tri-Carb liquid scintillation counter for a total of 5,000 counts. The results were expressed in terms of an aliquot of the injected solution analyzed simultaneously.

CALCULATIONS

When a trace quantity, M, of a substance normally present in the blood is injected intravenously, the tracer molecules become mixed with the native molecules present in the blood and in the interstitial fluid. Under certain conditions the tracer molecules eventually become evenly mixed with all of the native substance present in the body in an exchangeable form. Although this completely uniform ratio of tracer to native molecules is theoretically unobtainable except in a closed system, for practical purposes an equilibrium distribution may be obtained because the rates of excretion and/or metabolic destruction are slow relative to the rate of equilibration.

If the rates of formation, A, excretion, U, and metabolic destruction, F, of the native substance remain constant during the period of observation, and if

$$A = U + F, \tag{1}$$

then the plasma concentration, P, of native substance will remain constant, as will VP, the total amount in the body, where V is its apparent volume of distribution (assuming that V does not change).

Following equilibration of injected tracer, the amount, M_t, of tracer remaining at any given time, t, will decline at a rate given by the turnover rate of native substance, A/VP. It is assumed here that no tracer returns to the pool once metabolized:

$$-dM_t/dt = \frac{A}{VP} M_t. \tag{2}$$

Integrating

$$\ln M_t = -\frac{A}{VP} t + C, \tag{3}$$

where C is the constant of integration. When $t = 0$, $M_t = M$. However, the extrapolated value (at $t = 0$) of M_t, M_o, is likely to be less than the injected dose, M, by a variable amount; this is due to the fact that the average plasma concentration of tracer during the period of equilibration (and therefore the amount excreted) is greater than if equilibration had occurred instantaneously. Let x designate the difference between the extrapolated intercept value, M_o, and the amount injected, M (which is the intercept value which would have obtained if equilibration had occurred instantaneously):

$$M_o = M - x. \tag{4}$$

Substituting in Equation 3, we have, for any time greater than the equilibration period:

$$M_t = (M - x)\, e^{-(A/VP)t}. \tag{5}$$

The apparent volume of distribution, V, is given by:

$$V = \frac{M_o}{p_o} = \frac{M - x}{p_o}, \tag{6}$$

where p_o is the intercept value of the plasma concentration of tracer. The plasma concentration of tracer at time t, p_t (where t is greater than the equilibration period), is

$$p_t = p_o\, e^{-(A/VP)t}. \tag{7}$$

Therefore, p_o and A/VP may be derived from observations of p_t. In order to calculate V it is necessary to have an estimate of x, as Equation 6 shows.

If the rate of excretion of native substance, U, is large with respect to the rate of metabolism, F, then a good estimate of x can be obtained by observing the excretion of tracer, as shown in the following derivation.

The rate of renal (or renal plus extrarenal) excretion of tracer, u_t, is determined by the fact that the specific activities (or, in the case of stable isotopes, the atoms per cent excess) in blood and urine are equal with the qualifications noted below:

$$\frac{u_t}{U} = \frac{p_t}{P}. \tag{8}$$

Substituting for p_t from Equation 7,

$$u_t = \frac{U}{P} p_o\, e^{-(A/VP)t}. \tag{9}$$

The total amount of tracer, S, excreted up to time t (provided that t is greater than the equilibration period) is therefore:

$$S = x + \int_o^t u_t = x + \frac{UVp_o}{A}\left[1 - e^{-(A/VP)t}\right]. \tag{10}$$

Dividing by M, the amount of tracer injected, we have

$$\frac{S}{M} = \frac{x}{M} + \frac{UVp_o}{AM}\left[1 - e^{-(A/VP)t}\right]. \tag{11}$$

This equation can be treated as a straight line function of two variables, *viz.*, S/M and $[1 - e^{-(A/VP)t}]$, following the suggestion of Berkson, Keating, Power and McConahey (10). The intercept is x/M. Knowing the ratio A/VP and the fraction S/M excreted at each time interval, it is therefore possible to plot these fractions against values of $[1 - e^{-(A/VP)t}]$ and obtain x/M as the intercept.

A further complication is introduced by the fact that tracer removed from the blood by the kidneys requires an appreciable time, d, to reach the bladder. The specific activity of urine collected at time, t, therefore reflects the specific activity of plasma at an earlier time, $t - d$.

$$\frac{u_t/U}{p_t/P} = \frac{p_{(t-d)}/P}{p_t/P} = \frac{p_o\, e^{-(A/VP)(t-d)}}{p_o\, e^{-(A/VP)t}} = e^{(A/VP)d}, \tag{12}$$

where u_t represents the observed rate of excretion of tracer at time, t, as distinguished from the true rate. The "mean urinary delay time," d, can thus be estimated by comparing simultaneous specific activity of blood and urine. Solving Equation 12 for d,

$$d = \frac{VP}{A} \ln \frac{u_t/U}{p_t/P}. \qquad 13)$$

The fraction of the injected tracer *collected* up to time, t, designated as S′/M, is:

$$\frac{S'}{M} = \frac{x}{M} + \frac{UVp_o}{AM}\left[1 - e^{-(A/VP)(t-d)}\right], \qquad 14)$$

where t is greater than the sum of the time for equilibration and the urinary delay time. After this interval, the quantity, x, will have been excreted and further excretion will follow Equation 9.

Thus, the steps in calculating V and F from the plasma and urinary curves of tracer are as follows:

1) The turnover rate A/VP is obtained as the slope of the line relating log plasma concentration to time using values obtained after equilibration.

2) The "delay time," d, is obtained from Equation 13 and averaged for each experiment.

3) Values of $\left[1 - e^{-(A/VP)(t-d)}\right]$ are plotted against S′/M, and the intercept x/M is found.

4) V is calculated from Equation 6.

5) The production rate, A, is obtained as the product of the slope, A/VP, by the pool size, VP.

6) The rate of metabolism, F, is calculated by subtracting the rate of urinary excretion, U, from A (Equation 1).[2]

The assumptions made in this treatment are: *1*) The amount of injected material is negligible in relation to the amount present in the body. *2*) The tracer molecules are handled in a manner identical to the native molecules. *3*) The volume of distribution remains constant during the period of sampling. *4*) The rates of formation, excretion and destruction of native substance remain constant. *5*) The rate of excretion is large in relation to the rate of metabolic destruction. *6*) Metabolized tracer does not return to the pool.

Under the conditions of our experiments, N^{15}-labeled urea and C^{14}-labeled urea both appear to fulfill all of these assumptions except the last: *1*) The dose of N^{15}-urea injected was about 1.5 per cent of the pool size in one experiment and less than 0.4 per cent in the other four. The dose of C^{14}-labeled urea injected was negligible in relation to the urea pool. *2*) Isotope effects with these labeled forms of urea can be predicted to be small (11) although no definitive data are available. *3*) Urea diffuses very rapidly and attains a constant volume of distribution (12). *4*) Since the plasma concentration of ordinary urea remained nearly constant in these experiments, and the rate of urea excretion showed only minor fluctuations (the maximum deviation from the mean for each experiment in any single period was 9 per cent and the average deviation was 4 per cent for all periods), the steady state conditions were apparently fulfilled. *5*) The rate of excretion of urea is considerably greater than the rate of metabolism. *6*) Although tracer atoms evidently returned to the urea pool in both the N^{15} and C^{14} experiments, the effect on the calculated rate of metabolism is to make it appear smaller than the true rate (see Discussion).

[2] U/A may also be obtained from the slopes of the lines obtained in Step 3. This method (10) gives greater weight to the urinary data than the method outlined above. Since the collection and timing of samples is considerably more reliable for plasma than for urine, we have employed the urinary data only to obtain an estimate of the quantity, x.

RESULTS

The experiments were of two types: *1*) those in which plasma and urine samples were obtained at intervals of one to three hours, from which the production rate of urea can be calculated; and *2*) those in which urine was collected for several days in order to determine the recovery of labeled material in the urine. One experiment with N^{15} combined the features of both types of study. The results of this experiment are shown in Table I. Following the injection of N^{15}-urea, labeled nitrogen atoms appeared in urinary ammonia and urinary undetermined nitrogen. The atoms per cent excess N^{15} in these fractions remained less than that in urinary urea. The fraction of the dose excreted as urea was 79.5 per cent, as ammonia, 0.4 per cent and as total nitrogen, 89.6 per cent.

More detailed analysis of these data as well as the other experiments follow.

1. Curve of plasma disappearance

Figure 1 represents the atoms per cent excess N^{15} or specific activity of C^{14} in blood urea for all of the experiments of the first type plotted on a semilogarithmic scale. The first samples were obtained at least 90 minutes after injection, as suggested by earlier studies (12, 13). In all but one experiment, the decline in "p" with time is linear on this logarithmic scale. These slopes were calculated by the method of least squares. However, in one individual given C^{14}, Subject E, the decline is not a simple exponential function but involves another slower component or components. This change in slope is probably due to the reincorporation of labeled atoms into newly formed urea. No attempt was made to calculate production rate in this experiment. Of the nine experiments illustrated in Figure 1, only

TABLE I

Labeled nitrogen in plasma and urine following intravenous injection of N^{15}-urea†*

Time	Serum urea N		Urine vol.	Urine urea N				Urine ammonia N				Urine total N			
	Conc.	N^{15}		Conc.	N^{15}	N^{15}	Cum. dose	Conc.	N^{15}	N^{15}	Cum. dose	Conc.	N^{15}	N^{15}	Cum. dose
hrs.	*mg. %*	*a %*	*ml.*	*mg. %*	*a %*	*mg.*	*%*	*mg. %*	*a %*	*mg.*	*%*	*mg. %*	*a %*	*mg.*	*%*
0	15.0														
			1,080	131	0.415	5.86	19.7	4.2	0.086	0.04	0.1	165	0.373	6.64	22.5
2.0	15.2	0.279													
			355	342	0.287	3.49	31.4	18.8	0.029	0.02	0.2	435	0.252	3.89	35.4
4.1	14.8	0.232													
			290	446	0.227	2.94	41.3	25.7	0.010	0.01	0.2	566	0.197	3.22	46.2
6.1	15.2	0.174													
			315	357	0.177	1.98	48.0	18.6	0.013	0.01	0.2	446	0.156	2.19	53.6
8	15.2	0.143													
			1,600	662	0.070	7.46	73.0	26.2	0.003	0.01	0.3	845	0.061	8.25	81.3
32	17.3	0.022													
			1,290	853	0.011	1.21	77.1	41.9	0.002	0.01	0.3	1,088	0.010	1.36	85.9
56	17.5	0.004													
			2,220	561	0.004	0.44	78.6	24.0	0.002	0.01	0.4	723	0.003	0.51	87.7
79	14.1	0.000													
			2,080	405	0.001	0.09	78.9	22.6	0.001	0.00	0.4	529	0.001	0.15	88.2
99															
			2,335	744	0.001	0.19	79.5	30.7	0.001	0.01	0.4	936	0.001	0.31	89.2
130	17.4	0.000													
			1,510	605	0.000	0.00	79.5	29.3	0.001	0.01	0.4	758	0.001	0.15	89.6

* Experiment A1: 100 mg. urea containing 60.9 atoms per cent N^{15} injected at time zero (normal male, 74.1 Kg.).
† N^{15} expressed as excess above control samples. Blood and urine samples were obtained simultaneously.

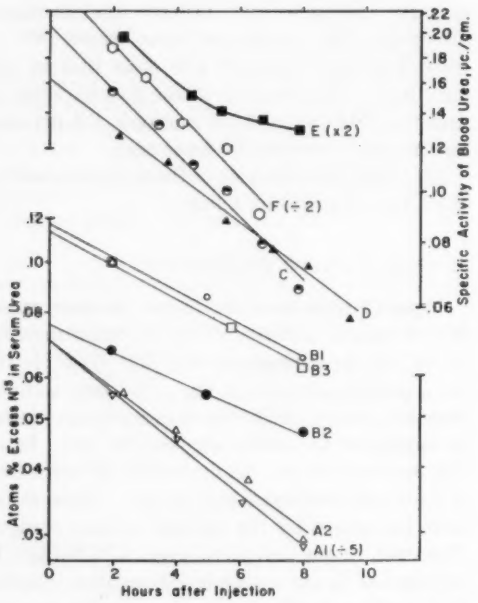

FIG. 1. THE RATIO OF LABELED TO UNLABELED UREA MOLECULES IN BLOOD AT INTERVALS FOLLOWING INTRAVENOUS INJECTION OF EITHER C^{14}-UREA (ABOVE) OR N^{15}-UREA (BELOW)

In Subjects E and F and in the first experiment in Subject A the results have been multiplied by constant factors in order to include them all in the same figure.

six were considered to fulfill the requirements for calculation of the rate of urea metabolism. In F, the urine samples were lost. In D, the plasma concentration of unlabeled urea exhibited a progressive decline during the experiment; consequently the steady state conditions were not fulfilled.

2. *"Urinary delay time"*

Inspection of the data in Table I reveals that the degree of enrichment of urinary urea nitrogen is greater than the average enrichment of plasma urea nitrogen during the same interval. The average plasma value, $\bar{p}/P$, may be calculated as follows:

$$\bar{p}/P = \frac{p_1/P_1 - p_2/P_2}{\ln (p_1/P_1)/(p_2/P_2)}.$$

The ratio of urinary urea enrichment (or specific activity) to plasma urea enrichment (or specific activity) has been calculated for the six technically satisfactory experiments. The results are shown in Table II. The proportion of labeled to unlabeled molecules is slightly greater in the urine than in the blood in all but one of the 19 periods. Using the measured slope of plasma disappearance (Figure 1), a "urinary delay time" may be calculated using Equation 13. The aver-

TABLE II

Apparent urinary delay time for urea

Expt.	Time	Ratio (u/U)/(p/P)	Delay	Urine flow
	hrs.		*hrs.*	*ml./min.*
A1	2.0–4.1	1.12	1.0	2.9
	4.1–6.1	1.13	1.1	2.4
	6.1–8.0	1.12	1.0	2.8
	8 –32	1.09	1.2	1.1
	32 –56	1.09	1.2	0.9
A2	2.2–4.0	1.07	0.6	5.0
	4.0–6.3	1.11	1.0	5.5
	6.3–8.0	1.14	1.2	3.3
B1	2.0–5.0	1.11	1.5	1.4
	5.0–8.0	1.10	1.4	1.0
B2	2.0–5.0	1.07	1.0	1.5
	5.0–8.0	1.00	0.0	3.4
B3	2.0–5.8	1.12	1.5	0.9
	5.8–8.0	1.13	1.6	5.5
C	2.0–3.5	1.16	1.1	3.7
	3.5–4.7	1.05	0.4	5.3
	4.7–5.7	1.09	0.6	9.8
	5.7–6.8	1.17	1.2	8.8
	6.8–8.0	1.19	0.6	3.5

age values for each experiment range from one-half hour to one and one-half hours. This finding was unexpected. Obviously a urea molecule does not require such a long period of time to pass from the glomerulus to the bladder. Furthermore, there is no clear relationship between delay time as calculated and the urine flow. Hence, this derived value does not represent delay due to the time required for passage of fluid from the kidney to the bladder. A systematic analytical error seems unlikely, since similar results were obtained with both C^{14} and N^{15}, employing different methods for urea determination. Undoubtedly, residual urine in the bladder contributes to the apparent delay in these experiments. But again the absence of a definite relationship between urine flow and apparent delay time indicates that this cannot be the only explanation. A more important factor may be the existence of a pool of urea in the kidney parenchyma with which the excreted urea is in equilibrium. A fall in the specific activity of filtered urea, therefore, would not be reflected in the excreted urea until the specific activity of this pool had reached the same value.

Whatever the explanation, the treatment which follows rests only upon the assumptions: *1*) that the specific activity of filtered urea was identical with that of plasma urea; and *2*) that the measured discrepancy between plasma and urine specific activity did not change between two and nine hours after injection.

An average value of "delay time" during the first eight hours following injection was calculated for each subject, as shown in Table III.

3. Calculation of excess tracer excreted during period of equilibrium

Using the slopes obtained from Figure 1 and the apparent delay times obtained from Table II, the values of the integral,

$$1 - e^{-(A/VP)(t-d)},$$

can be calculated and plotted against the fraction of the dose excreted, as in Equation 14. The resulting graphs are shown in Figure 2. For each subject a straight line is obtained. The intercepts vary from zero to 20 per cent of the dose. This variation presumably reflects the variable quantities of tracer reaching the kidney during mixing in various experiments. Being

TABLE III

*Urea production and metabolism in normal subjects**

Expt.	Wt.	Slope A/VP	Excess excretion x/M	Delay time d	Urea space V	Pool VP	Production A	Excretion U	Metabolism F	Ratio F/A
	Kg.	*hr.*$^{-1}$	%	*hr.*	*L.*	*Gm.*	*Gm./hr.*	*Gm./hr.*	*Gm./hr.*	
A1	74.1	0.114	13	1.0	44.9	14.5	1.64	1.29	0.37	0.20
A2	75.5	0.105	16	1.0	43.2	18.7	1.96	1.53	0.43	0.21
B1	62.7	0.070	00	1.4	38.0	13.8	0.96	0.75	0.22	0.23
B2	59.8	0.060	05	0.5	41.8	20.0	1.19	1.04	0.15	0.13
B3	60.2	0.075	04	1.6	40.2	13.0	0.98	0.62	0.36	0.36
C	48.5	0.134	19	0.9	27.4	12.1	1.62	1.30	0.32	0.20

* Subjects A and B received N^{15}-urea; Subject C received C^{14}-urea. Antibiotic preparation was as follows: A1, B1 and C, none; A2, succinylsulfathiazole 12 Gm. per day for five days followed by chlortetracyline 3 Gm. per day for three days; B2, oxytetracycline 2.25 Gm. per day for three days; and B3, succinylsulfathiazole 12 Gm. per day for six days.

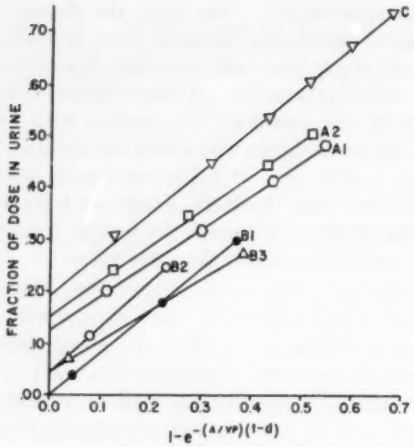

FIG. 2. THE FRACTION OF THE DOSE OF LABELED UREA RECOVERED IN THE URINE PLOTTED AGAINST A TIME INTEGRAL WHICH REPRESENTS THE FRACTION ANTICIPATED FROM THE PLASMA DISAPPEARANCE

Subjects A and B received N^{15}-urea; Subject C received C^{14}-urea. The intercepts represent the excess excretion of tracer during the period of equilibration.

highly diffusible, labeled urea may, to a large extent, be localized to the systemic organ it reaches on the first circulation, at least for several minutes. If this organ happens to be the kidney, a large fraction will be excreted during the period of equilibration. If it is some other organ, only a small fraction may reach the kidney during this time.

4. Calculation of volume of distribution, pool size, production rate and the rate of metabolism

Using the parameters obtained in the previous sections and the equations presented above, one obtains the derived values shown in Table III for the six technically satisfactory experiments, in three subjects, two of whom received N^{15}- and one C^{14}-urea.

The calculated urea space varies from 56 per cent to 70 per cent of body weight. These values are within the normal range for total body water as a fraction of body weight.

The urea pool of the body varies from 13 to 20 Gm. The rate of production varies from 0.96 to 1.96 Gm. per hour, and the rate of metabolism from 0.15 to 0.43 Gm. per hour. Obviously, all of these values, with the possible exception of urea space, may be expected to vary in a single individual, depending upon protein intake, water turnover and other factors.

The important data in Table III are contained in the last column which shows the ratio of urea metabolized to urea produced. In the control experiments the ratio was 0.20, 0.23 and 0.20 in the three subjects. Following administration of tetracycline derivatives and/or the unabsorbable sulfonamide, succinyl-sulfathiazole, no consistent changes in this ratio were observed in Subjects A or B. This result is compatible with the urea recovery data presented below which indicate that these drugs did not eliminate bacterial ureolysis in the gut. Unfortunately no technically satisfactory experiments of the production rate type were obtained during intestinal bacteriostasis with neomycin.

The data presented in Table III indicate that approximately one-fifth of the urea being synthesized in normal subjects is continually being destroyed. Ninety-five per cent confidence limits for the estimates of production rate in Experiment C, calculated by the method of Halperin and Walser (14) are 1.49 and 1.84 Gm. per hour. Since the rate of excretion was 1.30 Gm. per hour, the data indicate a significant difference between urea production and excretion, as determined by this statistical test. In the other two control experiments the number of plasma samples obtained was three and four, respectively; consequently application of statistical methods is probably unwarranted.

It may be noted that the calculation of urea space involves the same complications and corrections as the calculation of urea production rate. Therefore, to obtain an accurate estimate of the volume of distribution of urea following a single intravenous injection of tracer, it is necessary to take into consideration both "urinary delay time" and the excess excretion during equilibration. It is possible that these same sources of error might affect the calculation of the volume of distribution of other substances following a single injection. A discussion of this general problem is outside the scope of this work.

5. The cumulative excretion of labeled urea following intravenous injection

In Table IV is presented the total recovery of labeled urea in one experiment with N^{15} and 14

TABLE IV

*The urinary recovery of labeled urea administered intravenously in normal subjects**

Subject	Therapy	Isotope	Collection	Labeled urea recovered	
				Untreated	Treated
			hrs.	%	%
A	None	N^{15}	155	80	
C	None	C^{14}	46	65	
D	None	C^{14}	72	85	
D	Neomycin	C^{14}	72		98
E	None	C^{14}	46	64	
E	Succinylsulfathizole	C^{14}	46		81
F	None	C^{14}	64	75	
F	Neomycin and chlortetracycline	C^{14}	43		83
G	None	C^{14}	72	77	
H	None	C^{14}	46	83	
I	Neomycin	C^{14}	72		98
J	Neomycin	C^{14}	96		92
K	Neomycin	C^{14}	72		93
L	Neomycin	C^{14}	72		89
M	Neomycin	C^{14}	72		102

* Neomycin was given 3 Gm. per day for two days; succinylsulfathiazole 12 Gm. per day for six days; and chlortetracyline 3 Gm. per day for three days.

experiments with C^{14}. The period of collection varied from 43 to 155 hours. An approximate estimate of the amount of N^{15}-urea excreted after the first two days may be obtained from examination of Table I. Here 4.1 per cent of the dose was excreted as urea after 32 hours and 2.4 per cent after 56 hours. The results with C^{14}-urea were similar. In Subject G, 7 per cent of the dose was recovered between 45 and 61 hours after injection and 1 per cent between 61 and 77 hours. In Subjects D and I, receiving neomycin, 1 per cent of the dose was recovered between 48 and 72 hours after injection. Presumably, then, the total recovery of labeled urea would have been somewhat greater in those subjects in whom collection was continued for only two days.

Nevertheless the data presented appear to establish: *1*) that 15 to 30 per cent of the labeled urea does not appear in the urine in control studies; and *2*) that following neomycin therapy, the recovery of labeled urea is significantly increased. Considering only the subjects who received neomycin alone, the mean recovery is 95 per cent.

6. The presence of reformed N^{15}-urea in plasma

It has been pointed out that reincorporation of labeled carbon or nitrogen atoms into urea is the chief complication of the calculation of production rate. Such reincorporation is itself evidence for metabolism, but the methods employed above do not distinguish between labeled urea which was injected as such and labeled urea molecules formed by reincorporation of labeled atoms into newly synthesized urea.

As an incidental finding in this study, it was found that N^{15}-urea molecules injected can be distinguished from urea molecules containing N^{15} formed anew. The basis of this procedure lies in the existence of three types of urea molecules: those containing two N^{14} atoms, one N^{14} and one N^{15} atom, and two N^{15} atoms. The statistical distribution of these three types of molecules depends upon simple probability in a random mixture. In normal urea, only a minute fraction of the molecules contains two N^{15} atoms. In urea containing 60.9 atoms per cent N^{15}, this type of molecule predominates. Therefore, following injection of labeled urea, the proportion of these molecules is higher than would be expected from a random distribution. Newly formed urea containing N^{15} is composed of all three types in random distribution. Therefore, as the turnover of labeled urea proceeds, the proportions of the three types approach a normal statistical distribution. By reacting urea directly with hypobromite, it is possible to determine the proportion of the three types of molecules present (15).

Urine was collected between three and 12 hours

after injection of 25 mg. of N^{15}-urea in Subject A. Urea was isolated by extraction of the moist residue with hot acetone. A portion of this material was allowed to react directly with hypobromite and another portion was analyzed after Kjeldahl digestion. The ratio of N_2^{29} to N_2^{28} in the two samples was 0.0580 and 0.0305, respectively. Using these data and the formula for statistical distribution of the three types of molecules, it is possible to calculate the proportion of enrichment due to injected tracer and the proportion due to resynthesized urea (15). This calculation indicates that 15 per cent of the N^{15}-enriched urea was newly formed, the remainder corresponding in statistical distribution to the injected material. The true rate of urea metabolism is therefore greater than indicated by the methods employed above, which fail to distinguish between injected tracer and reformed labeled urea.

DISCUSSION

These experiments indicate that in man, as in animals, a considerable fraction of synthesized urea is continuously being degraded by intestinal bacteria. Several lines of evidence are presented in support of this conclusion: *1*) The estimated rate of urea production exceeded the measured rate of urea excretion by approximately 20 per cent; *2*) the recovery of labeled urea in the urine following intravenous injection was incomplete; *3*) 10 per cent of the excess N^{15} appeared in urinary ammonia and nonurea, nonammonia nitrogen following administration of N^{15}-urea in one experiment; *4*) a significant fraction of the labeled urea molecules in plasma following N^{15}-urea administration was found to be newly synthesized; and *5*) the recovery of labeled urea in the urine became nearly complete following oral administration of neomycin.

These data do not permit a precise estimate of the fraction of synthesized urea which is destroyed. The reappearance of labeled nitrogen or carbon atoms in newly formed urea tends to reduce the apparent rate of urea metabolism. The magnitude of this error is difficult to estimate. In the case of N^{15}, it is clearly considerable. The only evidence for a similar reincorporation of C^{14} atoms consists of the slow component in the disappearance of C^{14}-labeled urea from the plasma and the urine. The rate of urea production estimated with C^{14}-urea in one subject was similar to that estimated with N^{15} in the other two. However, one would expect labeled nitrogen precursors to gain access to synthesized urea much more readily than labeled carbon dioxide.

The mathematical procedure employed to calculate production rate tends to minimize the estimated rate, in comparison with other alternative calculations. For example, if the extrapolated plasma specific activity at the time of injection is employed to compute the urea space and urea pool, neglecting the excess tracer excreted during equilibration, higher values for production rate are obtained in five of six experiments. If the "apparent urinary delay time" is overlooked, the calculated production rate is again greater. The average ratio of 0.2 for urea metabolized to urea synthesized in the control studies therefore represents a minimum; the true value of this ratio may be considerably greater.

Since urea recovery following neomycin administration was not quite complete, the possibility remains that a small portion of the observed urea metabolism is due to other metabolic transformations. Kornberg and Davies (5) have demonstrated the absence of urease activity in mammalian tissues other than the gut, and have also calculated that the rate of spontaneous urea hydrolysis is insufficient to account for appreciable breakdown. Other conceivable metabolic pathways for urea include reversal of urea synthesis (1) and transamidation with arginine or creatinine. Creatinine was isolated (16) from the urine obtained between three and 12 hours after injection of 25 mg. of N^{15}-urea in a normal subject. The enrichment of creatinine nitrogen was 0.002 atoms per cent excess compared with 0.058 atoms per cent excess in urea excreted during the same interval. Thus, there is no evidence that urea undergoes any metabolic transformations in the body other than hydrolysis within the gut. The small amount of labeled urea not recovered following neomycin may have been excreted in the sweat or in the stool.

It has been established that the removal of ammonia from the blood is defective in the presence of liver disease (17), and that intestinal bacteriostasis diminishes the toxic symptoms of patients with impending hepatic failure (18). It

is therefore of some interest to calculate the amount of ammonia released into the portal venous blood by urea hydrolysis. The estimated rate of urea breakdown in the three control studies averaged 0.3 Gm. per hour or 7.2 Gm. per day. This is equivalent in nitrogen content to 11 Gm. per day of NH_4Cl. It is possible that the major beneficial effects of neomycin therapy in cirrhotics are attributable to reduction of bacterial urease.

The observations reported are also pertinent to the use of urea as a measure of total body water. The requirements of an ideal substance for measuring total body water include the absence of metabolic breakdown. The assumption that urea is not metabolized underlies the use of large doses of ordinary urea to measure total body water (19). The demonstration of significant breakdown of urea in man appears to invalidate this method but does not necessarily invalidate the use of labeled urea for this purpose. If the extrapolated plasma concentration at time zero is a true measure of the concentration which would have obtained had equilibration been instantaneous, then the derived value for urea space is an accurate one, whether or not metabolic breakdown occurs. The problem is, therefore, that of obtaining a reliable estimate of M_o, the amount of labeled urea which corresponds to the extrapolated plasma concentration at time zero. As pointed out above, the principle uncertainties are the excess urinary excretion during equilibration and apparent urinary delay. If both of these phenomena are neglected in calculating urea space, that is, if the observations on urine are omitted entirely, the estimated urea space would be zero to 20 per cent greater in the six studies illustrated in Figure 1. These corrections are therefore necessary when the tracer is administered by single intravenous injection. When these corrections are made, the calculated urea space should provide an accurate measure of the true volume of distribution of urea.

It remains to be demonstrated that the concentration of urea in body water is, for practical purposes, uniform. Although there are specialized phases of body fluids with different urea concentration such as the cerebrospinal fluid, the aqueous humor, the renal medulla and the epithelium of the urinary tract, the volume of these phases is too small to introduce significant errors into the estimation of total body water with labeled urea. Of greater importance is the urea concentration in the major portion of cell water. Although reliable measurements are not available, it is generally held that urea is freely diffusible in the water of body cells, with the above exceptions.

It may be concluded, therefore, that labeled urea deserves further trial as a measure of total body water. In subjects in whom the disappearance of labeled material from blood is a simple exponential function, a reliable estimate of urea space may be obtained. The identity of urea space and total body water is likely, but remains to be conclusively demonstrated.

SUMMARY

Urea labeled with N^{15} or C^{14} was injected intravenously into normal subjects and its disappearance from blood and urine was observed. The calculated rate of urea production exceeded the measured rate of urea excretion by 20 per cent. Approximately one-fourth of the labeled urea was not recovered in the urine. The true rate of urea breakdown is probably greater, since evidence was obtained that labeled atoms of N^{15}, and possibly C^{14}, were reincorporated into urea. Following oral neomycin administration, the urinary recovery of C^{14}-labeled urea became nearly complete.

It is concluded that at least one-fourth of synthesized urea is continuously being hydrolyzed by intestinal bacteria in normal subjects.

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THE LYSIS OF ARTIFICIALLY INDUCED INTRAVASCULAR CLOTS IN MAN BY INTRAVENOUS INFUSIONS OF STREPTOKINASE * †

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Streptokinase has been shown to be an effective therapeutic agent, effecting the rapid lysis of extravascular fibrin clots in man (1). As a result, extensive clinical studies of *extravascular* clot lysis have been made in a variety of disease states (2, 3). In the present studies, streptokinase was found to be equally effective in the lysis of artificially induced *intravascular* clots in man, when administered intravenously under precisely controlled biochemical conditions.

Recent investigations have indicated that streptokinase reacts with a proenzyme in plasminogen to form an activator complex (4–6). This complex, designated "activator" reacts with plasminogen (the proteolytic precursor) to form a proteolytic enzyme, plasmin (7, 8). In order to be effective in man, this proteolytic system must be uninhibited, or "free." The system may be interfered with at three levels. Streptokinase may be neutralized by its specific antibody and, therefore, be unavailable for activating the system; "activator" may be neutralized by inhibitor found in the plasma; and plasmin may react with still another inhibitor in the plasma (9–12).

Previous clinical studies of *intravascular* fibrin clot lysis for the therapy of thromboembolic disease in man have utilized streptokinase to activate the patient's plasminogen *in vivo,* or purified plasminogen previously activated *in vitro* (13–16). Interpretations of these earlier clinical studies are complicated by variations in the natural course of disease and by an anti-inflammatory effect which may follow clinical use of the enzymes (13, 17, 18).

In the present investigation, reproducible data were obtained by inducing experimental thrombi in human volunteers by direct irritation of the intima with a dental broach or by chemical irritation with sodium morrhuate. The position and size of the thrombi were determined by clinical observation and by venograms. The proteolytic system in blood believed to be responsible for physiological clot lysis (plasmin) was then activated by infusing streptokinase (SK) into a contralateral extremity at varying time intervals after clot formation.

Three different systems were established by varying the amount of SK infused: *1*) moderate amounts of circulating plasmin with no measurable free SK (*Method P*); *2*) negligible plasmin with moderate amounts of free SK or activator (*Method SK*); *3*) small amounts of both free SK and plasmin (*Method SK-P*). Since preliminary experiments had indicated that clot lysis was frequently followed by reformation of the clot at the same site, each system was evaluated for both its ability to lyse the experimental thrombi and prevent reformation of the thrombi.

In order to produce a fibrinolytic system by any of the three systems, it was found to be necessary to give an initial, or *priming dose,* sufficient to just neutralize the circulating SK antibody and inhibitor in each patient. Additional infused SK was then free to produce active proteolysis. This additional SK was infused in a *first sustaining dose,* in an amount appropriate for the production of one of the three proteolytic systems enumerated above, and these conditions were maintained *in vivo* for 24 to 36 hours by the continuous infusion of a *second sustaining dose* of SK. When thrombolysis had occurred, reformation of the clot was

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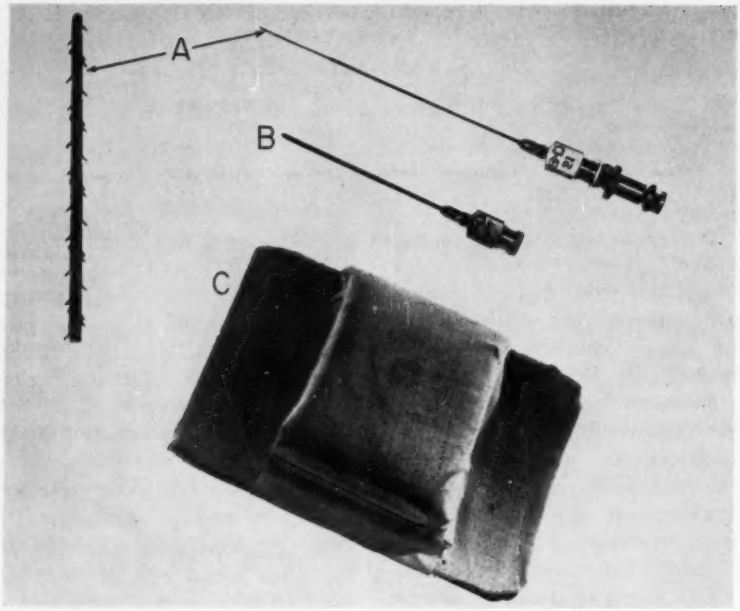

FIG. 1. APPARATUS FOR INDUCTION OF BLOOD CLOTS IN PERIPHERAL VEINS

A) Dental broach soldered in tip of two inch, No. 21 needle and photomicrograph of dental broach showing blunt tip and barbed area. B) Thin-wall No. 18 needle for venotomy and introduction of dental broach. C) Sponge rubber pad backed with stainless steel, for local application of pressure on proximal portion of vein.

prevented in those patients with sufficient residual plasminogen by prolonging the SK infusion for an additional four to six hours.

The amount and duration of the SK infusions necessary were determined primarily by following the various biochemical components of the streptococcal fibrinolytic system in the patient's blood before, during and after the infusions. By this means, it was possible to establish minute to minute control of the proteolytic conditions, making frequent changes of the infusion rate to maintain an appropriate balance of plasmin, plasminogen, plasmin-activator, SK and prothrombin. A balance was found to be necessary to lyse intravascular clots consistently and reproducibly and to prevent reformation of the clots.

A further check was provided by determining the presence or absence of the clot at frequent intervals by inspection, palpation and X-ray venograms of the affected extremity.

MATERIALS AND METHODS

Induction of standard thrombus. The blunt tip of a coarse dental broach [1] was soldered in the end of a two inch No. 21 needle [2] after the point had been cut off at right angles to the needle shaft (Figure 1). The broach was introduced into the lumen of the vein through a thin-walled No. 18 needle[3] and the shaft of the No. 21 needle rotated to the right and left through 360° for five minutes. At the same time, the No. 18 needle was moved back and forth on a longitudinal axis to the vein and digital pressure was applied to the vein wall overlying the dental broach. These measures served to insure irritation of the intima over the entire area.

Immediately after the needle was removed, local pressure was applied and a foam rubber pad (Figure 1)

[1] Dental Broaches, No. 6, extra coarse; Charles B. Schwed Company, New York, N. Y.

[2] Hypodermic needle, 21 gauge, two inch length; Becton, Dickinson and Company, Rutherford, N. J.

[3] Hypodermic needle, 18 gauge, thin wall, one and one-half inch; Becton, Dickinson and Company, Rutherford, N. J.

was loosely bound (with an elastic bandage [4]) proximal to the affected portion of the vessel for 18 to 24 hours to slow the rate of flow, fix the clot and prevent embolization.

A firm occluding clot was formed in less than 15 minutes. Blood clots 5 to 20 cm. long were produced by this method in the superficial saphenous veins or the veins of the forearm. A few clots were produced by means of a sclerosing agent, sodium morrhuate.[5] The technique was an adaptation of one previously used (19) and was found to be too complicated for use in man.

The presence or absence of the clot was determined by inspection, palpation and X-ray venograms taken before, during and after the SK infusion. Repeated venograms were made with diatrizoate sodium [6] diluted to 13 per cent in saline to prevent irritation of the intima. At least two X-rays were taken for each venogram. One view was made with a retrograde pressure of 40 mm. of mercury proximal to the clot and the other was made without retrograde pressure. In both instances, a standard pressure of 110 cm. of water was used to inject the diatrizoate sodium.

Streptokinase. Lots 21A, 22A and 23A of concentrated, highly purified streptokinase (SK) were used.[7] Each contained approximately 600 units of SK per μg. of nitrogen and was virtually free of streptococcal desoxyribonuclease.

Preparation and rate of infusions. Streptokinase for intravenous use was dissolved in a solution containing 5 per cent dextrose and one-half per cent human serum albumin.[8] As a *priming dose,* 1 to 2 ml. of a solution containing 5,000 units per ml. was infused each minute until 100,000 to 1,000,000 units was administered. As a *sustaining dose,* 2 ml. of a solution containing 240 to 320 units per ml. (depending on the method used) was infused per minute for approximately 24 hours. Since the infusion rate was very critical, specific variation from this will be considered in individual protocols.

Clinical observations. During the period of the infusion, pulse, temperature and blood pressure were taken every hour or when specifically indicated, every 15 minutes. The thrombosed vessel was examined hourly by at least two observers. After the infusion, the observations were generally continued for 24 to 48 hours.

Anti-pyretic medication. If a temperature elevation to 102° F. or more occurred, four doses of amidopyrine (0.6 Gm.) and chlorprophenpyramine maleate [9] (4 mg.) were given during the subsequent 24 hour period as indicated previously (20).

ANALYTICAL METHODS

In order to compare fibrinolytic or inhibitor activities, each unit was based upon the amount required to lyse a standard clot in 10 minutes, except for plasminogen, which was 30 minutes. Thus, these activities were interconvertible.

Streptokinase. The fibrinolytic assay for streptokinase was performed by a modification of the method of Christensen (21) as previously described (19).

Streptokinase-activated plasmin. Logarithmic dilutions of plasma were added to the standard clot system used above (19) except that a lot of bovine fibrinogen [10] was used which was found to be low in contaminating plasminogen and was further purified by the method of Laki (22).

A modification of the method of Schwert and Takenaka (23) was also employed for the quantitative determination of plasmin, utilizing a final concentration of 0.002 M benzoylargenine methyl ester.

A qualitative fibrinolytic test for SK-plasmin was also performed by the addition of fresh undiluted whole blood to a previously siliconed [11] graduated centrifuge tube fitted with a wire retainer for the formed clot. The "per cent lysis" as described previously (20) was easily obtained by this method. When the "per cent lysis" was less than 25 per cent, it was arbitrarily considered to be due to clot retraction. With one exception, in this laboratory, the "per cent lysis" (or clot retraction) of normal controls has been less than 20 per cent in more than a thousand determinations.

SK-activator complex and/or free SK. Dilutions of patient plasma were added to an excess of plasminogen and tested as in the SK assay. The fibrinolytic activity of the contained plasmin (as determined separately) was subtracted from the total, the remainder representing activator and/or free SK. Since the assay did not distinguish between activator and free SK, they will not be differentiated further in this study.

Streptokinase antibody and SK-plasmin inhibitor. Because the measurement of SK antibody and SK-plasmin inhibitor presented a number of special problems, biochemical and immunologic studies on them will be presented in detail in a subsequent publication. The SK-plasmin inhibitor under consideration here was found to be comparable with the fast-acting alpha-2 inhibitor of Jacobsson (10) and Norman and Hill (11). Measurements of antiplasmin were also made, but were not reported here since they did not appreciably influence the amount of proteolysis induced during the infusion of SK.

Each unit of antibody and inhibitor represents the inhibition of one unit of SK in the standard SK assay. Since the inhibition reaction was not temperature-dependent, preincubation of the test substance with SK was

[4] Ace Bandage®; Connecticut Bandage Mills, Inc., Bridgeport, Conn.

[5] Sodium morrhuate 5 per cent with benzyl alcohol 2 per cent; G. D. Searle and Company, Chicago, Ill.

[6] Hypaque® brand of diatrizoate sodium, Winthrop Laboratories, New York, N. Y.

[7] The highly purified streptokinase was supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

[8] Normal serum albumin (human), salt-poor, U.S.P., Cutter Laboratories, Berkeley, Calif.

[9] Chlor-Trimeton Maleate® brand of chlorprophenpyramine maleate, Schering Corporation, Bloomfield, N. J.

[10] Bovine Fibrinogen, lot No. N1803, The Armour Laboratory, Kankakee, Ill.

[11] DC 200, Dow-Corning Corporation, Midland, Mich.

TABLE I

Streptokinase antibody and SK-plasmin inhibitor in the circulating blood of patients prior to the intravenous infusion of SK

Patient	Antibody	Inhibitor	Total inhibition (antibody and inhibitor)
Cra	40,250	43,050	83,300
Pab	52,700	61,000	114,000
Dav	63,000	79,000	142,000
O'To	58,000	89,000	147,000
Moo	85,000	145,350	230,850
Nic			290,000
Jon	153,000	147,000	300,000
McD	132,000	189,000	321,000
Gle	173,000	177,000	350,000
Jac	202,000	248,000	450,000
Bor	225,000	245,000	470,000
Tur	470,000	70,000	540,000
Bla	160,000	410,000	570,000
Sve	340,000	245,000	585,000
Mus	380,250	302,250	682,500
Total = 15	Mean 194,977 Range 40,250–470,000	Mean 175,046 Range 43,050–410,000	Mean 354,670 Range 83,300–682,500

carried out for 10 minutes at 4° C. in order to minimize the activation of contaminating plasminogen. In practice, the inhibition of one-half of a unit was determined and the value then doubled. At least 10 tubes were used for each assay with six dilutions and a one unit and two one-half unit controls. A means of separating antibody from inhibitor activity was developed when it was found that strong chelating agents such as disodium ethylenediamine tetraacetate [12] or an ion exchange resin [13] removed SK-plasmin inhibitor activity but had no effect upon the SK antibody. Both activities are present in serum. Therefore, the SK-plasmin inhibitor was estimated by subtracting the antibody value found in plasma from the total inhibition in the serum.

The inhibitor and antibody obtained by these methods were calculated per ml. of plasma. These values, in turn, were multiplied by the estimated plasma volume to determine the total circulating antibody and inhibitor for each patient.

Plasminogen. The fibrinolytic assay for plasminogen was performed as described previously (19).

Fibrinogen. Blömback and Blömback's (24) method was employed. The isolation procedure was performed with ice cold reagents, in an ice bath. Considerable emphasis was placed on clot formation at an optimal concentration of fibrinogen. The optical density at 282 mμ, $E_{1\ cm.}^{1\ per\ cent}$, was found to be approximately 16.17 for fibrinogen in alkaline urea solution as previously determined by Blömback.

Complement. The serum complement was determined by the method employed by Fischel, Pauli and Lesh (25).

Prothrombin. A one-stage determination was utilized for the prothrombin time with each of the two thromboplastins [14] used. The second is stated to be free of proconvertin-like activity (26).

[12] Versene® brand of disodium ethylenediamine tetraacetate, Versenes, Inc., Framington, Mass.

[13] Dowex 50-X®, Dow Chemical Corporation, Midland, Mich.

RESULTS

A. Biochemical rationale

Phase I. Neutralization of antibody and inhibitor. In previous reports (20, 27), it was concluded that circulating SK antibody and SK-plasmin inhibitor(s) must be neutralized by the injection of at least an equivalent amount of SK to establish a fibrinolytic system *in vivo*. Further studies seemed to indicate that the fast acting inhibitor of Jacobsson (10) was primarily responsible for inhibition of the activation of SK-plasmin *in vivo*. Therefore, antibody and inhibitor determinations were made on both the patient's plasma and serum prior to the infusion of SK.

The amounts of antibody and inhibitor found in most of the patients included in this study are tabulated in Table I. Those samples tested by a less accurate method developed earlier are omitted. The mean antibody level was 194,977 units and the range was 40,250 to 470,000 units. The mean inhibitor level was 175,046 units and the range was 43,050 to 410,000 units. It is evident that the range of each of these was very wide indeed

[14] Simplastin®, Warner-Chilcott Laboratories, Morris Plains, N. J. and Acuplastin® brand of thromboplastin extract, OrthoPharmaceutical Corporation, Raritan, N. J.

and prior determination of these values is essential for the estimation of an appropriate priming dose of SK to neutralize the antibody and inhibitor *in vivo*.

Strong evidence was obtained that the priming dose necessary to produce detectable fibrinolytic activity could be based upon the total inhibition present in the blood. The infusion of an amount of SK equal to the SK antibody in the plasma produced no detectable fall in the circulating plasminogen. An amount of SK equal to, or just greater than, the combined antibody and SK-plasmin inhibitor (100 per cent on the abscissa, Figure 2) produced a barely detectable change in the circulating plasminogen. The results were the same in patients treated to produce an excess of plasmin (*Method P*) or free SK (*Method SK*). Direct measurements showed that little or no plasmin was formed during Phase I and neutralization was virtually complete. These plasmin measurements were corroborated by studies of the lysis time of the patients' whole blood when clotted *in vitro* as for a Lee-White clotting time, by one-stage prothrombin-time determinations (Figure 3) in which the destruction of accelerator globulin or prothrombin occurs in the presence of free plasmin (20, 28, 29) and by measurement of the fibrinogen and complement levels of the plasma (20, 30). The extent and mode of participation of antibody and inhibitor, respectively, in this neutralization, are not clear.

In a few instances where the infusion rate for the priming dose was very rapid, the resulting high concentration of SK produced an increase in the velocity of the enzyme-substrate reaction and therefore a fall in plasminogen which was relatively independent of the antibody titers in the peripheral blood. This sequence of events is illustrated in Table II, when 572,000 units of SK was given within a period of one hour and 12 minutes.

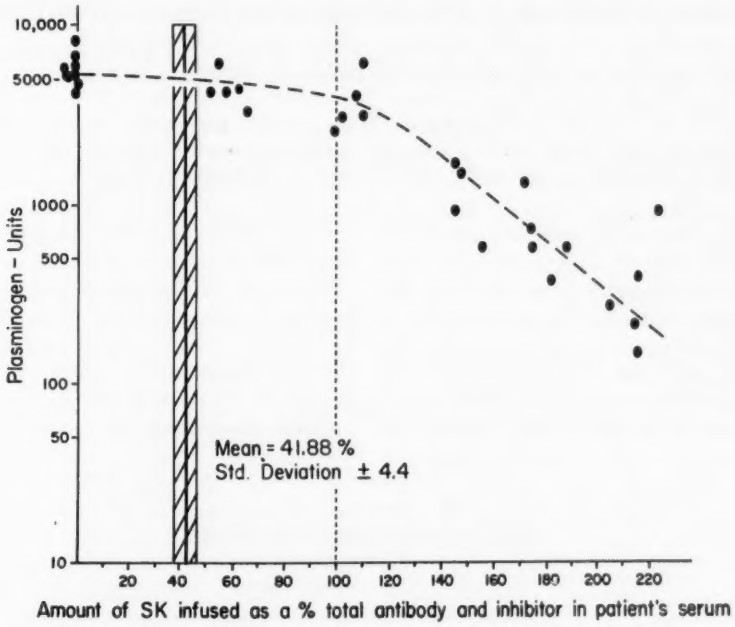

FIG. 2. EFFECT OF INTRAVENOUS INFUSION OF PRIMING AND FIRST SUSTAINING DOSE OF STREPTOKINASE ON PLASMINOGEN LEVELS OF PATIENTS TREATED ACCORDING TO *METHOD SK-P* (SEMI-LOG PLOT)

Dashed line represents a mean of values. At cross-hatched line, 41.88 per cent, amount of SK infused is equal to mean of total antibody in patient's serum; at dotted line, 100 per cent, amount of SK infused is equal to total antibody plus inhibitor in patient's serum.

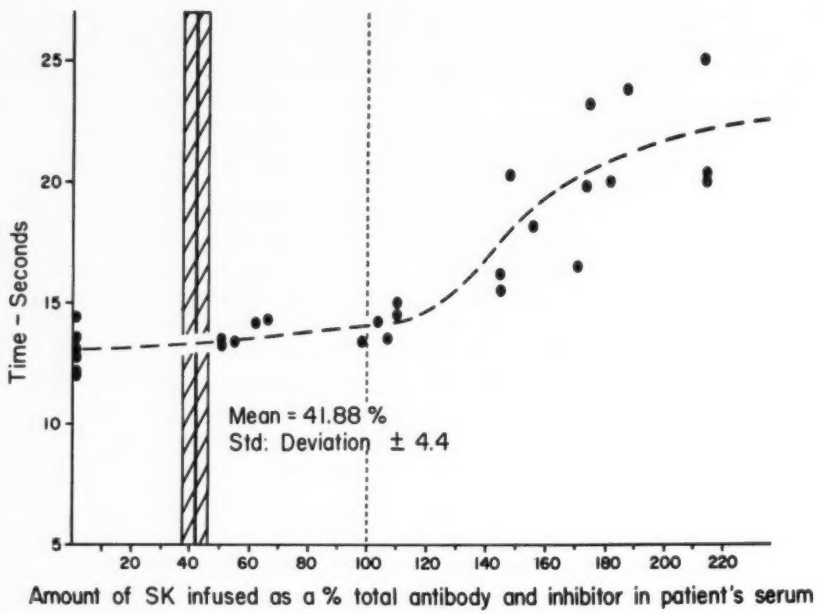

FIG. 3. EFFECT OF INTRAVENOUS INFUSION OF PRIMING AND FIRST SUSTAINING DOSES OF STREPTOKINASE ON THE PROTHROMBIN TIME, AS DETERMINED ON PLASMA OF PATIENTS TREATED ACCORDING TO *METHOD SK-P*

Dashed line represents a mean of values. At cross-hatched line, 41.88 per cent, amount of SK infused is equal to mean of total antibody in patient's serum; at dotted line, 100 per cent, amount of SK infused is equal to total antibody plus inhibitor in patient's serum.

Rarely, during a moderately rapid infusion of the priming dose (20,000 to 30,000 units per minute), the plasminogen, antibody and inhibitor fell slowly concurrently, during the entire period of Phase I. When the infusion rate was very slow (less than 500 units per minute), SK antibody fell first, then inhibitor and finally, plasminogen. These data were attributed to differences in the rate of association of SK and antibody, activator and inhibitor and activator and plasminogen.

Phase II. Initiating a fibrinolytic system. Following neutralization of the antibody and inhibitor, additional SK was infused as a *first sustaining dose,* in an amount appropriate for the production of each of the three proteolytic systems.

Small amounts of SK (about 5,000 units per

TABLE II

Effect of the intravenous infusion of the priming dose of streptokinase on the circulating antibody, inhibitor and plasminogen levels in a patient

		Antibody	Inhibitor	Total inhibition	Plasminogen	Cumulative SK dosage
	Bla*					
(Pre)	11:15	160,000	410,000	570,000	6,200	
	11:23	Start of SK infusion				
	11:33	207,000	178,000	385,000	5,400	100,000
	11:45	218,000	148,000	366,000	5,200	212,000
	12:03	165,000	75,000	240,000	69.5	360,000
	12:35	12,000	11,000	23,000		572,000

* Patient Bla was infused rapidly at the rate of 10,000 units per minute.

hour) produced moderate or large amounts of plasmin (*Method P*). The plasminogen level was only moderately reduced by this procedure from 5,000 units to 2,000 or 3,000 units per ml. of plasma.

Large amounts of SK (200,000 to 400,000 units per hour) produced a very rapid fall in plasminogen to levels of less than 100 units, and free SK or activator was evident in the blood (*Method SK*). No free plasmin could be detected.

With intermediate amounts of SK (about 100,000 units per hour for two to three hours) the plasminogen fell rapidly to levels of 200 to 500 units (Figure 2) and small amounts of plasmin and free SK were released into the circulation (*Method SK-P*).

Phase III. Sustaining the fibrinolytic system. SK was infused for periods up to 48 hours in Phase III to sustain the fibrinolytic system according to one of the three methods outlined below. In those patients treated according to *Method P,* Phase II merged imperceptibly with Phase III. Thus, the injection of very small amounts of SK (25,000 to 50,000 units per day) produced moderate levels of plasmin in the circulating blood for as long as three to five days. As a result, lysis of the patient's clotted whole blood was effected *in vitro* within two to three hours, the one-stage prothrombin time was markedly elevated, and the fibrinogen was moderately or markedly depleted with potentially serious effects to the patient. In two patients, the prothrombin time went up to 50 seconds, the fibrinogen was no longer detectable and a few small areas of petechiae and ecchymosis appeared on the skin.

In patients treated by *Method SK,* free SK was maintained in the circulating blood for long periods of time by a sustained, continuous infusion of 45,000 to 60,000 units per hour. Since the patient's plasminogen was already low, this excess SK drove the level down further to less than 100 units per ml. of plasma. When rapidly induced, little free plasmin was produced by this method, the coagulation constituents were not markedly depleted and the prothrombin time rarely rose to more than 23 to 24 seconds.

In *Method SK-P,* an attempt was made to generate small amounts of both SK and plasmin in the circulating blood. The infusion rate ranged from 20,000 to 45,000 units per hour, varying from 30,000 to 45,000 units per hour in high antibody, high inhibitor patients to 20,000 to 30,000 units in low antibody, low inhibitor patients.

The rate of infusion, to provide free SK without excessive depletion of the endogenous plasminogen, was very critical. This was necessary to provide sufficient enzyme for the lysis of the secondary clots which tended to reform on the matrix of the original clot. In practice, when the plasminogen level was maintained at approximately 100 to 200 units per ml. of plasma, clot lysis generally occurred without clot reformation. At plasminogen levels below 100 units per ml., the initial clot generally lysed but tended to reform. At plasminogen levels above 300 units per ml. the initial clot was markedly resistant to lysis. Therefore, an effort was made to maintain the plasminogen level at approximately 100 to 200 units until the clot was lysed (Figure 4).

The infusion rate during Phase III varied for each patient, and in addition, had to be changed during the infusion period. In general, a steady state was maintained for only eight to 10 hours when the plasminogen level began to go down very slowly. This could be compensated for by decreasing the rate of infusion by about 2,500 units of SK per hour, at appropriate time intervals.

The importance of this decrease in plasminogen was brought to our attention on several occasions by palpable evidence of clot reformation concurrent with a temporary, minor fall in plasminogen (below 100 units). The clot which reformed usually lysed again when the SK infusion was *immediately* decreased by as little as 2,500 to 5,000 units per hour. It is significant, however, that when clot reformation followed a *sustained* reduction in plasminogen, the absence of adsorbed plasminogen on the fibrin clot made it almost impregnable to further treatment.

Although the plasminogen assay is extremely sensitive, has a very wide range and indicates activator activity of plasminogen, it became increasingly evident that it was not sufficiently sensitive to be used as the only guide to the infusion rate in the fine balance of enzyme and substrate necessary in this phase.

Plasmin and free SK assays were also used in an attempt at a more precise regulation of the infusion rate. However, the difficulties indicated in Materials and Methods limited their usefulness.

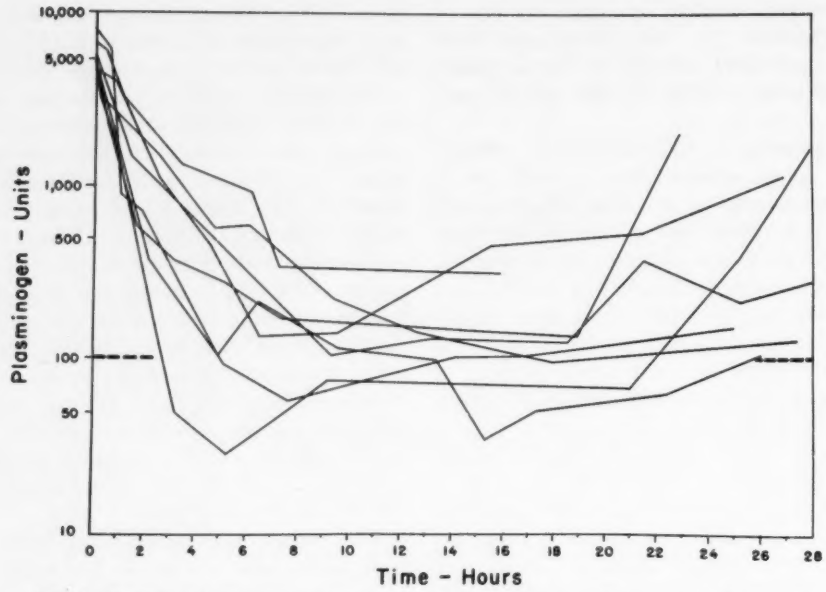

FIG. 4. EFFECT OF INTRAVENOUS INFUSION OF STREPTOKINASE ON PLASMINOGEN LEVELS OF PATIENTS TREATED ACCORDING TO *METHOD SK-P*

For emphasis, an abscissa was drawn through 100 units of plasminogen on the ordinate.

The one-stage prothrombin test has been mentioned as an indicator of plasmin activity in Phases I and II. When the test is performed by one individual, a variation of only two seconds in a sequential test may become an important, early guide toward a change in the infusion rate. After a steady state has been reached, a two second increase in the prothrombin time may denote excess plasmin formation, while a comparable decrease may indicate excess activator or SK in the circulating blood.

When a clot had been lysed, the infusion rate became much less critical. There was very little "need" for free SK, and the SK infusion rate was decreased very gradually to allow the plasminogen to increase to 200 or more units (Figure 4). The infusion was continued at this rate for four to six hours longer to minimize the danger of excessive plasminogen depletion with clot reformation and to provide more plasmin for the lysis of secondary clots.

Illustrative case: Method SK-P. The dynamic interaction of the components of the fibrinolytic system may be seen in data from Patient BOR, given 2,145,000 units of SK according to *Method SK-P,* over a period of 31 hours (Figure 5). His circulating blood was estimated to contain approximately 225,000 units of antibody and 245,000 units of inhibitor, a total of 470,000 units of inhibition.

Two blood clots were induced in Patient BOR by the dental broach technique. A clot 10.5 cm. long formed in the forearm veins of the left arm 48 hours prior to the infusion. A clot 15 cm. long formed in the forearm veins of the right arm 24 hours prior to the infusion of SK. The position and size of the clots were documented by venograms.

In Phase I, a priming dose of 500,000 units of SK was infused over a 25 minute period. As a result, the plasminogen, complement and fibrinogen levels decreased slightly. When the patient's whole blood was allowed to clot *in vitro,* about 75 per cent lysis occurred in 24 hours, although no lysis was evident in eight hours. The prothrombin time was normal and the plasmin and free SK assays were negative. It would appear that neutralization of the antibody and inhibitor was barely complete at the end of the priming

dose, a marginal amount of plasmin was produced, and the streptococcal fibrinolytic system was ready for activation.

In Phase II, 150,000, 100,000 and 75,000 units of SK were injected rapidly as "stat" injections during a continuous infusion of 32,000 units per hour. The plasminogen level promptly decreased from 6,000 units to about 100 units, five to six units of plasmin and one unit of free SK appeared; the prothrombin time rose from 14 to 20 seconds and the patient's per cent lysis increased to 100 per cent in two hours. Thus, an effective fibrinolytic system was produced during Phase II, with a demonstrable amount of free SK and plasmin.

In this patient, and indeed, in most of the patients treated according to *Methods SK-P* and *SK*, the fibrinogen and complement levels remained about the same during this phase. This was interpreted to mean that plasmin was destroyed almost as quickly as it was formed, and being present only transiently, affected only the preferential substrates, plasminogen, accelerator globulin and prothrombin. Rapid administration of moderate amounts of SK was essential for this prompt elimination and control of the formed plasmin. In other patients, when SK was given too slowly during Phase II, plasmin was not rapidly destroyed and fibrinogen, complement and prothrombin were seriously depleted.

In Phase III, the infusion rate was increased to 40,000 units per hour for about five hours. During this period the 24 hour clot softened perceptibly, decreasing from 15 cm. in length to about 5 cm. The infusion rate was then arbitrarily increased to 50,000 units per hour in an attempt to reverse the lytic process. Shortly after the fifteenth hour, the plasminogen level dropped to 50 units and the clot reformed (at arrow, Figure 5). The infusion rate was promptly decreased to 45,000 units, then to 40,000 units, when thrombolysis started again. This type of experiment was performed repeatedly. As indicated previously, when the infusion rate was not decreased immediately, the clot resisted all further efforts to lyse it.

About 24 hours after the start of the infusion, when the clots were thought to be lysed, the infusion rate was decreased to 35,000 units per hour and maintained at that level for approximately six additional hours. It may be noted that the plasminogen, plasmin, free SK, prothrombin time, fibrinogen and complement were maintained at relatively constant levels during Phase III. The only change was seen in the per cent lysis which

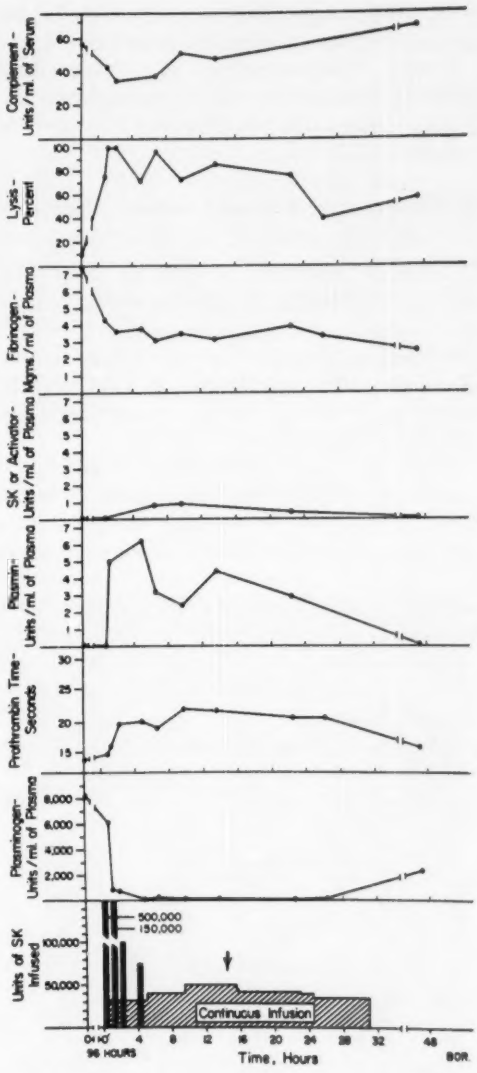

FIG. 5. EFFECT OF INTRAVENOUS INFUSION OF 2,145,000 UNITS OF STREPTOKINASE IN PATIENT BOR, TREATED ACCORDING TO *METHOD SK-P* FOR 31 HOURS

Ninety-six hours elapsed between the time preinjection samples were taken and the beginning of streptokinase infusion. Solid bars represent rapid injection of a major part of priming and first sustaining doses of SK.

went down progressively as the amount of free plasmin decreased during the infusion.

Clinical observations and X-ray venograms, made on Patient BOR the day following the infusion, revealed that both clots had lysed and did not recur.

B. The lysis of artificially induced blood clots in vivo

Standard blood clots, 5 to 20 cm. long, were artificially induced in the large superficial veins of the forearm or the superficial saphenous veins of volunteers as described in the section on Materials and Methods. Thirty-two clots were induced in 26 patients by a "closed" technique utilizing a dental broach.

Three untreated control clots produced by the morrhuate technique and 10 by the dental broach technique went on to eventual fibrosis. None spontaneously lysed within three or more weeks of observation. Five were excised three to five weeks after they were formed and gross and histological section showed them to be fibrosed.

In six patients, two clots were induced, one 48 hours and one 24 hours, respectively, prior to the SK infusion.

The total dose and duration of each of the SK infusions in the patients treated according to *Methods P, SK* and *SK-P* are summarized in Table III.

In those patients treated by *Method P* the total amount of SK infused averaged about 85,000 units and ranged from 50,000 to 150,000 units (Table III). Since intermittent, small doses of SK sufficed to maintain a fibrinolytic system in these patients for long periods of time, the duration of the infusion was comparatively short, averaging only six hours. Patients with low antibody and inhibitor were generally selected for this, since a relatively "pure" plasmin system could be readily maintained in this type of patient over a 24 hour period with only 25,000 to 50,000 units of SK.

An exception to this occurred with one patient in this group (Table III) who had a streptococcal infection for about two weeks prior to the infusion of SK. His circulating antibody and inhibitor at the time he volunteered for the study were within normal limits. However, his immune response increased rapidly after the infusion was started, presumably because of a "booster" response. Consequently, the infused SK was rapidly neutralized, only a very small amount remaining for the production of plasmin. In spite of receiving a total dose of 3,680,000 units in 26 hours, his plasminogen level never went below 2,000 units from his normal of 5,200 units. Insufficient SK was available to overcome the immune response at this time.

The total amount of SK given to the patients, treated according to *Methods SK* and *SK-P*, averaged 1,928,500 and 1,466,125 units, and the mean duration of the infusions was 29 and 26.5 hours, respectively (Table III).

The X-rays in Figures 6 and 7 illustrate the course of a typical patient treated according to *Method SK-P*. A thrombus was induced in the patient's right arm 48 hours prior to the infusion and in the patient's left arm 24 hours prior to the infusion. Venograms made on the right arm (Figure 6b) and on the left arm (Figure 7b), immediately prior to the infusion, showed that the previously patent vessels were completely occluded. The 48 hour clot (Figure 6b) was esti-

TABLE III

Total dose and duration of the SK infusion in patients treated according to Methods P, SK and SK-P

Method	No. in group	Total dose of SK (average)	Total dose of SK (range)	Duration of infusion (average)	Duration of infusion (range)
		units	*units*	*hrs.*	*hrs.*
P	5*	85,000*	50,000–150,000*	6*	3–9
SK	5	1,928,500	1,050,000–2,764,000	29	21–40
SK–P	8	1,466,125	715,000–2,145,000	26.5	7–48

* One patient, who fulfilled the biochemical requirements for inclusion in this group, with an excess of SK-plasmin, was omitted from this tabulation. This patient had a streptococcal infection for about two weeks prior to the infusion and a rapidly rising titer of SK antibody after the infusion was started. He received a total dose of 3,680,000 units of SK over a period of 26 hours.

mated to be 20 cm. long and the 24 hour clot (Figure 7b) about 15 cm. long, by both clinical and radiological criteria. SK was infused over a period of 36 hours by *Method SK-P* (1,805,500 units). The 24 hour clot lysed completely in 18 hours, but the 48 hour clot did not lyse until nearly 30 hours. The infusion was continued for an additional six hours to prevent reformation of the clot. X-ray venograms made the following morning confirmed the fact that clot lysis had occurred (Figures 6c and 7c). These clots did not reform.

Relative effectiveness of Methods P, SK and SK-P

Table IV summarizes a number of the experiments.

In seven instances, SK was infused according to the method which produced circulating plasmin (*Method P*) without detectable free SK. Partial lysis of the clots occurred in three and complete lysis in one, but all four clots reformed. Thus, no persistent clot lysis occurred.

SK was infused in seven instances according to the method which produced circulating free SK (*Method SK*) without demonstrable amounts of plasmin. Two clots lysed and did not reform, but complete or incomplete lysis was followed by reformation of the clot in three. No lysis was evident in two clots. Persistent clot lysis occurred in only two of seven clots treated by this method.

SK was infused in 11 instances according to the method which produced small amounts of circulating free SK and plasmin (*Method SK-P*). Complete, persistent clot lysis occurred in all. It is evident that consistent and reproducible results were obtained with *Method SK-P* only.

In no instance did persistent thrombolysis of 48 hour clots occur unless *Method SK-P* was used.

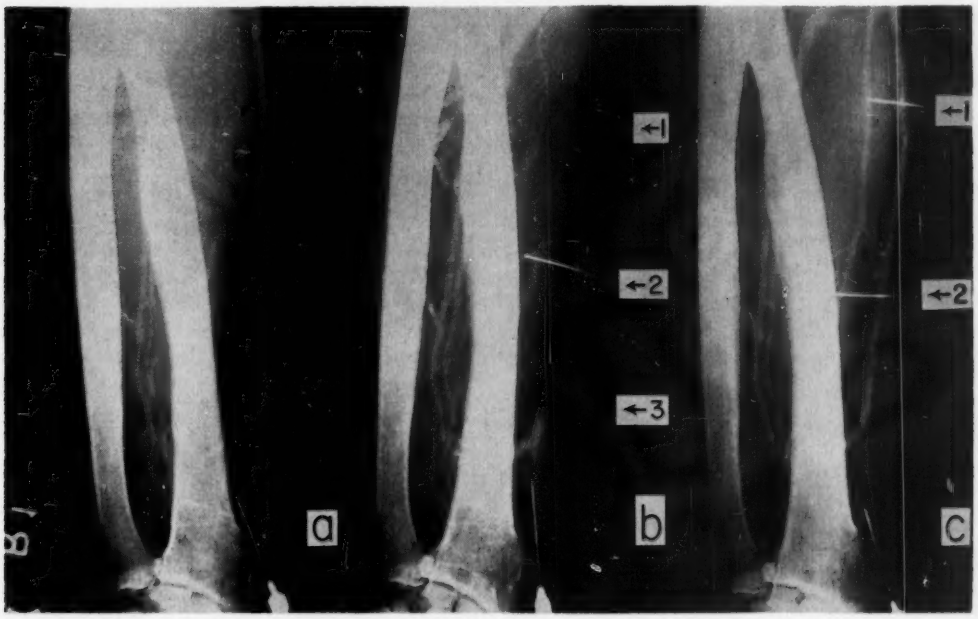

FIG. 6. X-RAY DEMONSTRATION OF CLOT LYSIS

To determine extent of clot lysis and reformation, venograms were made on Patient G's right arm: *a*) prior to induction of clot, as a control; *b*) 48 hours after clot was induced and prior to infusion of SK, defining the clot; and *c*) 24 hours after the SK infusion, showing that complete lysis had occurred, without clot reformation. Radiopaque lines at **1** and **2** were made by nichrome wires on skin surface to define original area of clot induction. Arrow at **3** indicates distal portion of the clot. Patient was treated according to *Method SK-P*, utilizing small amounts of plasmin and free SK. Lysis of the clot occurred after 30 hours of infusion.

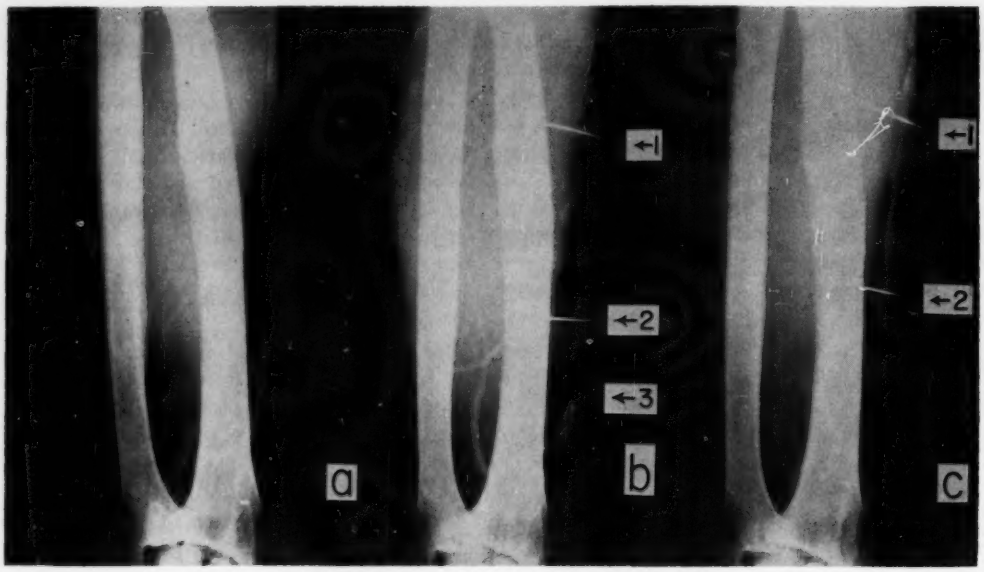

FIG. 7. X-RAY DEMONSTRATION OF CLOT LYSIS

To determine extent of clot lysis and reformation, venograms were made on Patient G's left arm: *a*) prior to the induction of clot, as a control; *b*) 24 hours after clot was induced and prior to infusion of SK, defining the clot; and *c*) 24 hours after SK infusion, showing that complete lysis had occurred, without clot reformation. Radiopaque lines at 1 and 2 were made by nichrome wires on skin surface to define original area of clot induction. Arrow at 3 indicates distal portion of the clot. Patient was treated according to *Method SK-P*, utilizing small amounts of plasmin and free SK. Lysis of clot occurred after 18 hours of infusion.

It was our impression that 48 hour clots took longer and were more difficult to lyse than 24 hour clots.

Toxicity

Toxicity studies on SK have been published previously (27, 31). In the course of the present study high purity streptokinase (600 units per μg. of N) was used exclusively. No hypotension was evident in any of the patients studied. An occasional rise in temperature, to 102° F., was immediately controlled by giving the patient amidopyrine and chlorprophenpyramine maleate as described in Materials and Methods. In one instance, the patient's temperature rose to 103° F.

There has been no evidence of embolic phenomena.

The patients treated by *Method P* presented a

TABLE IV

Lysis of intravascular blood clots in man by the intravenous infusion of streptokinase

Method of therapy	No. of clots	Age of clot before infusion		Clot lysis effected					No lysis effected
		24 hrs.	48 hrs.	Partial	Complete	Clot reformed	Lysis persisted	Total	
P	7	6	1	3	1	4	0	4	3 (1–48 hrs.)
SK	7	4	3	3	2	3 (1–48 hrs.)	2	5	2 (2–48 hrs.)
SK-P	11	8	3	0	11	0	11	11 (3–48 hrs.)	0
Total	25	18	7	6	14	7	13	20	5

potential hazard when plasmin was in great excess and the fibrinogen, prothrombin and accelerator globulin were severely depleted. Mild ecchymosis and petechiae formed in the skin of two of these patients.

The patients treated by *Methods SK* and *SK-P* formed little plasmin when SK was injected rapidly in Phase II and were remarkably well controlled in this respect. The prothrombin time remained under 26 seconds and rarely went higher than 24 seconds. No ACTH or cortisone has been used, or found to be necessary in these patients; an observation in contrast to the findings of other workers (16).

One severely hypertensive volunteer had a previous history of bouts of hypertensive encephalopathy with flaccid upper and lower extremities on the left lasting for several days. A single 24 hour clot was induced. At the end of his SK infusion, according to *Method SK-P,* the dose of SK was decreased, his prothrombin time returned to normal, the per cent lysis was within normal limits, and no plasmin or activator was found in the circulating blood. After the infusion, he developed a severe hypertensive encephalopathy similar to the previous episodes, but this time his neurological signs gradually progressed. Two days later he died. On autopsy, a firm blood clot was found around the lenticulostriate artery with no evidence of clot lysis. Microscopic section showed a rupture of the lenticulostriate artery. Thus, although a break occurred in the vessel wall, there is no evidence to suggest that this system affects the continuity of the blood vessels. Since there was no fibrinolysis in the peripheral blood of this patient at the onset of the encephalopathy, and no fibrinolysis in the clot adjacent to the lenticulostriate artery at autopsy, this patient's death was thought to have no relation to the previously induced fibrinolytic system. Indeed, a controlled fibrinolytic system has been induced over 150 times in patients in this hospital without serious toxicity.

On the other hand, the case history presented here emphasizes the need for great care in the selection of patients, with special reference to a possible or potential break in the continuity of the vascular endothelium.

DISCUSSION

The present study has demonstrated for the first time, so far as the writers are aware: *a*) that consistent and reproducible intravascular clot lysis can be produced in man; *b*) that this may be produced by activating the naturally occurring fibrinolytic enzyme of human plasma with SK; and *c*) that this same system may be used to prevent reformation of the clots.

Although the observations reported here are limited in number, they acquire broader significance from previous studies in animals conducted in this and other laboratories, where it was shown that fibrinolysis could be produced by an excess of activator or SK without excessive plasmin formation (19, 29, 32). *In vitro* studies by Ratnoff (33), Müllertz (34) and Alkjaersig, Fletcher and Sherry (35) indicate that fibrinolysis is produced more readily by activator or SK than by plasmin alone.

Since clot lysis has been produced in animals with trypsin (29) and preformed plasmin (14, 36), it was desirable to compare the relative efficacy of a fibrinolytic system utilizing excess plasmin with a fibrinolytic system utilizing an excess of free SK or activator, under comparable conditions, in man.

The data show that *in vivo* clot lysis occurred when an excess of free SK or activator was produced (*Methods SK* and *SK-P*), but did not occur when plasmin was in excess (*Method P*). Thus effective clot lysis is presumed to be due to free SK or activator. Since the assay used did not differentiate the two, and because human plasminogen is thought to contain the precursors for both activator and plasmin (7, 8), clot lysis could result from the combination of adsorbed plasminogen and *1*) circulating free SK or *2*) activator, or *3*) both.

The relatively high levels of plasminogen which are present with *Method P* militates against the formation of free SK with this system, but is entirely consistent with the formation of activator. The large amount of plasmin that is produced also indicates that plasminogen has been activated. However, it is not certain that activator is stable at these low concentrations of SK. Therefore, the fact that little or no clot lysis occurred when *Method P* was used, may be due to the qualitative

inefficacy of plasmin and activator as fibrinolytic agents, or a quantitative insufficiency of free SK and activator.

The very low levels of plasminogen available for activator formation with *Methods SK* and *SK-P*, which appear to be necessary for effective clot lysis *in vivo*, suggest that fibrin lysis was caused by free SK in excess of that which combined with plasminogen to form activator and plasmin in the circulating blood. Although the available evidence favors free SK, it is entirely circumstantial in nature.

In preliminary experiments, reformation of clots induced by intimal irritation usually took place immediately after clot lysis had occurred and the infusion was discontinued, indicating that intimal repair was probably necessary to prevent reformation of the clot.

Since clot reformation had been prevented in animals (19) by prolonging the infusion of SK, it was decided to use the SK-plasmin system as *1*) a fibrinolytic agent and *2*) an anticoagulant.

In *Method P*, the excess plasmin depleted the accelerator globulin and prothrombin, thereby acting as an anticoagulant. Unfortunately, thrombolysis was inadequate in patients treated by this method.

When an excess of free SK was produced (*Method SK*), little plasmin was formed, the prothrombin time remained under 25 seconds and anticoagulant action was limited. Therefore, the plasminogen on the clot and free SK in the blood were utilized to produce continuous fibrin lysis, the clot constantly reforming and lysing until intimal repair was effected. This was only partially successful in that most of the clots lysed, but most of them also reformed in patients treated by this method. It was concluded that the initial lysis of the clot had occurred because plasminogen had been adsorbed on the clot when it formed (prior to the infusion). As soon as the circulating plasminogen decreased to less than 100 units, adsorption of plasminogen on the reforming clot became limited and further lysis was prevented.

In *Method SK-P*, the amount of free SK and plasmin produced were small, but the plasminogen level was maintained at 100 to 200 units until persistent clot lysis occurred. There was little evidence of clot reformation because lysis of the clot probably occurred simultaneously, in dynamic equilibrium with clot reformation. Intimal repair was completed by prolonging the infusion of SK four to six hours after clot lysis became evident, clinically.

The following hypothesis was formulated on the basis of the studies previously mentioned (19, 29, 32, 35) and the data presented here:

> Circulating plasminogen is adsorbed on the fibrin clot as it forms. Adsorption effectively removes the plasminogen from association with, and the effect of, circulating inhibitors of both plasmin and activator. SK is infused in amounts calculated to neutralize the circulating antibody and fast-acting inhibitors. Additional infused SK, circulating free in the blood stream, combines specifically with its substrate plasminogen which had been previously adsorbed on the clot. Fibrinolysis is produced.
>
> The intimal damage to the vein wall persists. Clot reformation tends to occur. As the fibrin clot reforms, it adsorbs all the constituents essential for its own destruction: plasminogen, SK and plasmin. Consequently, fibrinolysis recurs. This sequence of events is thought to continue until intimal repair is effected, preventing further reformation of the clot.

Thus it is concluded that a fibrinolytic system with an excess of free SK or activator (*Methods SK* or *SK-P*) will consistently produce clot lysis and will be an effective therapeutic tool if it is modified to prevent clot reformation as well (*Method SK-P*).

Studies are now in progress to determine whether anticoagulants such as heparin can be used in man as ancillary agents in the anticoagulant phase of clot lysis. Previous experiments in animals indicated they could, when given for 24 hours or more (19).

The experimental data presented here, although limited, strongly suggest that 48 hour clots are initially more difficult to lyse and reform more rapidly than 24 hour clots, and are therefore in basic agreement with the studies of Back, Ambrus, Simpson and Shulman (37).

Toxicity due to streptokinase has been markedly decreased since high purity SK became available. Thus, hypotension following its administration has virtually disappeared and pyrogenicity is readily controlled by antipyretic agents. There is, however, the possibility of serious extravascular hemorrhage from vascular dysfunction or trauma during the SK infusion, despite the fact that the coagulation system is not seriously depleted when the patient is treated according to *Method SK-P*.

Although the therapeutic potential of intravenous SK or plasmin remains to be explored on a much wider basis (13–16), the results presented here clearly indicate that the fundamental assumptions implicit in the therapeutic application of intravenous SK or SK-plasmin are correct. SK will consistently lyse clots and prevent reformation of the clots when infused intravenously under carefully controlled biochemical conditions. The critical nature of these conditions, however, requires simpler methods for the evaluation of the fibrinolytic system and an approach to the technique of infusion which will create these conditions in any individual by the simultaneous administration of one or more agents to lyse a clot, prevent it from reforming, and diminish the potential hazard from systemic fibrinolysis.

SUMMARY

1. Large amounts of high purity streptokinase (SK) have been infused intravenously to produce intravascular clot lysis in man under sharply defined conditions.

2. Thirty-eight blood clots, 5 to 20 cm. long, were induced in the peripheral veins of volunteers by direct irritation of the intima with a dental broach or by chemical irritation with sodium morrhuate. No spontaneous lysis occurred in 13 controls. The position and size of the clots were documented clinically and by venograms. In the experimental group, SK was infused intravenously into a contralateral extremity 24 or 48 hours after production of the clot.

3. Three methods have been developed and tested for their fibrinolytic potential *in vivo*. The methods produced:

A) large amounts of circulating plasmin (*Method P*);

B) large amounts of free SK or activator (*Method SK*);

C) small amounts of plasmin and free SK or activator (*Method SK-P*).

In *Method P*, a priming dose of SK is calculated from the quantitative determination of the antibody and inhibitor in each patient's circulating blood to neutralize his antibody and inhibitor. Additional infused SK is free to produce an active fibrinolytic system. Small, intermittent doses of SK, 25,000 to 50,000 units per day, suffice to produce large amounts of plasmin.

SK was infused in seven instances according to *Method P*. There was no persistent clot lysis. Although minimal lysis was present in three and complete lysis occurred in one, all the clots reformed. Circulating plasmin was present in all seven without detectable free SK.

In *Method SK*, the priming dose of SK neutralizes the patient's circulating antibody and inhibitor as in *Method P*. The infusion of a first sustaining dose of 100,000 to 300,000 units of SK in one to three hours usually serves to reduce the plasminogen sharply and protect the patient by preventing the production of excessive amounts of plasmin. The continuous infusion of 45,000 to 65,000 units per hour provides large amounts of free SK or activator. When SK was infused in seven instances, according to *Method SK*, two clots lysed without reforming. Incomplete or complete lysis was followed by clot reformation in three (presumably due to a deficiency of circulating plasminogen). No lysis occurred in two.

In *Method SK-P*, the priming dose and first sustaining dose of SK are infused as in *Method SK* above. The second sustaining dose of SK is infused at 25,000 to 45,000 units per hour to provide plasmin and free SK (or activator) without excessive depletion of plasminogen. For this reason, the rate of infusion for *Method SK-P* is necessarily very critical. Frequent determinations of the patient's plasminogen, plasmin, free SK, plasmin-activator and prothrombin time assisted in maintaining this critical infusion rate.

In 11 instances SK was infused according to *Method SK-P*. Persistent clot lysis was obtained in all 11.

4. Forty-eight hour clots were more resistant to lysis than 24 hour clots regardless of the method used.

5. No embolic complications have been observed.

CONCLUSIONS

The results demonstrate that SK will consistently and reproducibly lyse intravascular clots and prevent reformation of the clots when infused intravenously into patients under precisely defined biochemical conditions.

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THE VARIABILITY OF EXTRACELLULAR FLUID SPACE (SUCROSE) IN MAN DURING A 24 HOUR PERIOD *†

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WITH AN APPENDIX

AN EXAMINATION OF A SEQUENCE OF RANDOMIZED ERRORS BY THE MONTE CARLO METHOD

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The water content of the human body averages 60 per cent of its total weight. Techniques for measurement and criteria for accuracy have been developed for total body water but there are no generally accepted standards for extracellular fluid volume (EFV). It is our purpose in this paper to describe our exploration of the erratic variations of extracellular fluid volume in normal subjects as measured by sucrose infusions and to discuss wide fluctuations in what had been thought hitherto to be a "steady state."

It is believed that most methods of estimating EFV using nonelectrolyte labels achieve rapid equilibration in, and measure mainly, interstitial and plasma water, and that the substances used diffuse incompletely into the cerebrospinal fluid, joint spaces, bone and tendon water, or into glandular lumina, or gastrointestinal tract water (2). This incomplete mixing of test substance in these spaces should not be expected to introduce large errors into this estimation since plasma and interstitial fluid must comprise the major portion of EFV. Walser, Seldin and Grollman (3) have proposed the term "functional EFV" for the volume thus measured. However, the accuracy of EFV estimates has been further questioned with reports that even large nonelectrolyte molecules, such as inulin and sucrose, may leave the EFV and penetrate cell membranes (4, 5).

Most methods for measuring extracellular fluid space are based on the assumption that concentrations of the labeling substance in plasma and interstitial pool are in equilibrium when the blood samples are taken. The chief criticism of the single injection technique is that such equilibrium cannot be achieved (6). This shortcoming of the single injection method was removed in a constant infusion technique described by Schwartz, Schachter and Freinkel (6). With this technique, equilibrium of the labeling substance in the "functional" extracellular fluid pool was achieved at the time of measurement. The proof of equilibrium was the demonstration of a constant value for the plasma concentration of the test substance when measured over several consecutive periods of approximately one hour each. A later modification, the "calibrated infusion difference" method described by Deane, Schreiner and Robertson (7, 8) offers the advantage of several EFV estimates in one study.

In a study of the distribution of body water, we decided to measure EFV by the calibrated infusion difference method using sucrose as the indicator. Our experiences with six hour sucrose infusions using this technique suggested that EFV varied from period to period more than has been described previously in control situations (7). To explore these variations, and to make a critical examination of the calibrated infusion difference technique, we decided to extend these infusions over 15 to 24 hour periods. We have attempted to estimate the error of the method and speculate on the significance of the observed EFV changes. Because of the popularity and

* A preliminary report has been published (1).

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convenience of the six hour infusion EFV measurement, we have compared the mean and variation of EFV in six hour studies with longer ones.

METHODS

A. Subjects. The subjects for these studies were volunteers. The majority were normal individuals (staff or student), but six studies were made on nonedematous hospitalized patients. The diagnoses of the patients are given in Table I.

B. Procedure. Since equilibrium between plasma and other extracellular fluids cannot occur if the intravenous infusion rates vary or are erratic, we used a calibrated delivery pump.[1] The pump motor moved a syringe plunger in a 1 ml. tuberculin syringe via an eccentric cam. The pump was adjusted to deliver a stroke volume of approximately 0.05 ml. and a minute volume of from 1.5 to 3.0 ml. Two counters built into the machine recorded the elapsed operating time in 0.1 second intervals and the number of times the syringe was emptied. The pump was calibrated for each study by pumping some of the infusion fluid into successive 25 ml. volumetric flasks for a period exceeding one hour. Under these conditions, the pump delivered a constant volume with a maximal error of ± 0.5 per cent and standard error of ± 0.3 per cent. The pump was fitted with small gauge polyethylene tubing (2.0 mm. O. D.) attached to the valve inlet and outlet. The inlet tubing was put directly into the sterile sucrose solution and the distal end of the outlet tubing was fitted by a plastic ferrule to a 20 gauge needle in a vein of the patient's forearm. The entire apparatus and tubing were sterilized before use by filling the system with aqueous Zephiran® (1:1,000) for at least 12 hours. Sterile water was used to flush the Zephiran® from the system just before priming with sucrose.

The entire apparatus was wheeled to the patient's bedside. The lightweight polyethylene tubing which carried the infusion fluid was seven feet long so the patient could move his arm during the infusion. A short polyethylene catheter for blood sampling was secured in the antecubital vein of the other arm.

The concentration of sucrose in the infusion fluid was approximately 5 per cent in water or, in a few instances, normal saline. Specimens for blank values were obtained for blood and urine within the hour immediately preceding the start of the infusion. The initial infusion rate[2] of 3 ml. per minute was maintained until after the first hour blood sample was collected. Then the rate was reduced and maintained constant at a value approximating half the initial rate for the rest of the study period. All samples were collected at approximately one hour intervals. Urine was collected by an indwelling Foley catheter, and the bladder was washed with three 75 ml. volumes of sterile saline at each collection. The beginning of the third saline bladder wash indicated the termination of one period and the beginning of another. At that instant the readings of the stroke and time counters were recorded. Five ml. oxalated blood samples were collected similarly at the beginning and end of each period. After the end of the infusion, the patient's urine was collected for another 24 hours. It contained most of the remaining sucrose.[3] All samples were refrigerated until analyzed, usually on the following morning.

C. Chemical methods. Filtrates of blood and urine were prepared by the barium hydroxide-zinc sulfate method of Nelson and Somogyi (10). The sucrose in blood plasma and urine was determined in duplicate by the resorcinol method of Roe as modified by Higashi and Peters (11). The blank values ranged from 1 to 2 mg. per cent sucrose equivalent for blood plasma, and from zero to 1 per cent for urine. In the studies reported in this paper, the analytical error of the various sucrose measurements was estimated from the mean difference in optical densities of the duplicate determinations. The standard error was essentially the same for blood and urine samples (2.0 per cent). The exact sucrose concentration of the infusion was found by analysis of an aliquot at the same time the blood and urine filtrates were analyzed.

To calculate sucrose space during the infusion, we used the calibrated infusion difference method described by Deane, Schreiner and Robertson (7) with slight modifications. At any one period the sucrose volume of distribution is $Vx = \frac{\Sigma iv - \Sigma uv}{P_t}$, where Vx is the sucrose volume of distribution, Σiv is the total amount of sucrose infused, Σuv is the total amount of sucrose excreted, and P_t the plasma sucrose concentration. Besides estimating sucrose space in this manner, we also calculated the space after adjusting Σuv for sucrose not recovered and P_t for plasma sucrose fluctuations. Complete recovery of infused sucrose was not always obtained, nor was a constant plasma concentration achieved consistently. Therefore, we corrected Σuv for unrecovered sucrose by dividing the measured Σuv by the per cent sucrose recovered, and by this maneuver adjusted Σuv to 100 per cent recovery (see Discussion for details and example). This correction assumed that the unrecovered sucrose disappeared at a rate which was a constant

[1] Brewer Automatic Pipetter, Model 40, Baltimore Biological Laboratories.

[2] This constituted the priming dose and differed from the method of Gaudino and Levitt (9) as well as from that of Schwartz, Schachter and Freinkel (6) in being an accelerated rate of infusion for one hour, rather than a single injection. The intent was to accelerate equilibrium as evidenced by a constant plasma sucrose level. The method of Deane, Schreiner and Robertson (7) did not include a priming dose.

[3] Urine collection of the test molecule after the end of the infusion was not used in calculation of EFV by Deane, Schreiner and Robertson (7), but was used by Schwartz, Schachter and Freinkel (6). We have used this urine sucrose recovery in the previously described manner (6) as well as in corrections for sucrose losses.

TABLE 1

Summary of 17 prolonged sucrose infusion studies

Study No.	Subject initials	Age	Height	Body wt.	Remarks	Duration of infusion	Post priming infusion rate	Average plasma sucrose level	Coeff. variat.* plasma sucrose	Average urine sucrose ex.	Coeff. variat. sucrose ex.	Duration of post-infusion recovery	Total sucrose recovered
		yrs.	*in.*	*Kg.*		*hrs.*	*mg./min.*	*mg. %*		*mg./min.*		*hrs.*	*as % infused*
31	R. P.–1	34	72	75	Normal	24	219.4	160.0	6.3	210.6	11.7	72	97.3
34	A. K.	26	67	55	Normal†	24	138.4	166.0	11.0	135.2	17.5	72	103.0
36	W. L.	32	71	86	Chronic meningoencephalitis	25	148.7	109.0	8.1	139.4	14.3	24	99.8
37	H. J.	66	72	64	Possible lateral sclerosis	24	167.8	164.5	17.8	140.8	14.0	48	93.0
38	W. F.	31	69	59	Multiple sclerosis	24	61.9	46.9	8.0	58.4	11.9	24	93.9
39	J. O'M.	34	66.25	75	Tension headaches	24	69.2	51.2	19.3	60.0	13.9	24	91.3
49	D. S.	29	67	78	Normal	17	28.0	20.5	9.6	23.4	16.6	24	86.0
51	C. P.	30	70.5	70	Normal	18	68.7	61.3	9.6	60.9	34.4	24	91.5
54	R. B.	25	69	63	Normal	18	82.3	61.9	13.9	72.4	10.0	24	91.4
55	L. M.	35	69.75	86	Angina pectoris	18	78.4	58.8	14.7	68.8	17.5	48	92.5
70	R. S.	35	73	83	Normal	14	103.0	84.6	8.6	82.8	25.5	24	83.4
73	R. H.–1	28	70	91	Normal	15	80.8	60.3	10.9	67.8	23.6	48	85.0
78	R. H.–2	28	70	91	Normal	15	72.1	57.2	7.3	63.5	20.6	48	97.6
88	R. H.–3	28	70	92	Normal	15	77.9	63.3	9.6	68.1	13.4	48	95.6
111	R. H.–4	28	70	96	Normal	14	96.0	75.7	13.6	79.7	18.5	24	91.7
125	R. P.–2	35	72	70	Normal	10	98.0	63.4	9.3	90.6	24.2	24	99.2
137	B. B.	28	71	87	Normal	14	100.4	64.9	10.5	76.9	19.9	24	85.9
Average				77.7					11.1		18.1		92.8

Study No.	Subject initials	Age	Height	Body wt.	Remarks	Sucrose space (corrected for recovery)				Final interval space uncorrected	Final interval space corrected for recovery	Post-infusion recovery space	Δ Corrected recovery space‡
						Mean	Range as % mean		Coeff. variat. 3–24 hrs.				
							Entire	Beyond 3 hrs.					
		yrs.	*in.*	*Kg.*		*as % body wt.*				*% body wt.*	*% body wt.*	*% body wt.*	
31	R. P.–1	34	72	75	Normal	23.1	29	20	4.3	28.9	22.3	21.6	0.6
34	A. K.	26	67	55	Normal†	23.1	59	45	13.1	15.7	22.0	22.6	−0.6
36	W. L.	32	71	86	Chronic meningoencephalitis	18.0	43	43	7.9	24.6	23.9	23.9	0
37	H. J.	66	72	64	Possible lateral sclerosis	29.3	28	28	9.7	38.4	28.2	26.1	2.0
38	W. F.	31	69	59	Multiple sclerosis	13.7	87	42	9.1	32.4	14.7	13.9	0.8
39	J. O'M.	34	66.25	75	Tension headaches	20.3	33	33	11.7	45.8	23.8	21.7	2.1
49	D. S.	29	67	78	Normal	25.5	28	28	9.0	46.8	22.2	20.2	1.9
51	C. P.	30	70.5	70	Normal	26.0	20	20	5.1	38.2	25.7	22.8	2.9
54	R. B.	25	69	63	Normal	19.6	42	42	11.9	34.3	18.5	17.2	1.3
55	L. M.	35	69.75	86	Angina pectoris	18.4	31	25	6.8	27.8	17.8	16.3	1.5
70	R. S.	35	73	83	Normal	20.1	55	47	16.7	35.9	17.9	16.3	1.7
73	R. H.–1	28	70	91	Normal	19.0	27	27	7.9	38.3	17.0	17.1	−0.1
78	R. H.–2	28	70	91	Normal	17.7	24	21	7.6	21.6	19.3	19.2	0.1
88	R. H.–3	28	70	92	Normal	17.8	16	11	3.7	29.0	18.5	17.0	1.5
111	R. H.–4	28	70	96	Normal	15.3	27	27	6.7	23.7	16.7	15.4	1.3
125	R. P.–2	35	72	70	Normal	19.5	20	18	5.0	22.4	21.4	23.3	1.8
137	B. B.	28	71	87	Normal	19.1	31	20	6.5	38.1	21.4	20.0	1.5
Average				77.7		19.4 (or 15.1 L.)	35.3	29.3 (or 4.4 L.)	8.4 (or 1.3 L.)	31.8	20.7	19.7	1.2

* Coefficient of variation = $\frac{\text{standard deviation} \times 100}{\text{mean}}$.

† Difference between final interval space corrected for recovery and postinfusion recovery space.

‡ Only woman.

fraction of the sucrose excreted in each period throughout the test and for the following 24 hours. A postinfusion sucrose recovery space also was calculated by dividing the postinfusion urine sucrose recovery by the final plasma sucrose level. This latter calculation did not contain the correction for incomplete sucrose recovery.

Because unexplained fluctuations occurred in plasma concentrations of sucrose throughout our studies we believed that a better approximation of the plasma concentration over any one period could be obtained by averaging the plasma sucrose concentrations of successive periods. Hence, P_t was obtained from $\frac{P1 + P2}{2}$, where P1 was the plasma level at the beginning and P2 at the end of a collection period. For simplicity, the correction for plasma water was not applied until the space calculation was made. The correction used was based on an assumed value of plasma water content of 92 per cent.

Throughout this paper the term "essentially constant" includes minimal changes that do not exceed the 95 per cent confidence interval for the factor under discussion. The 95 per cent confidence intervals of the various aspects of the technique are as follows: *1)* Blood sucrose, standard error [4] is equal to 2 per cent so that a 95 per cent confidence interval on measured value is approximately 96 per cent to 104 per cent, with a range of 8 per cent; *2)* urine sucrose, standard error is ± 2 per cent and 95 per cent confidence interval is ± 4 per cent; *3)* sucrose space,[5] standard error is ± 2.7 per cent or 95 per cent confidence interval is ± 5.5 per cent. We interpret changes exceeding the 95 per cent confidence limits in any factor between intervals as evidence of physiologic changes.

RESULTS

The range and frequency of sucrose space variations

Sucrose infusions were continued for over 24 hours in six subjects (see Figure 1), for 18 hours in four subjects and for 10 to 15 hours in seven (Table I). Sucrose space was calculated at hourly intervals, giving a mean sucrose space for the entire group of 19.4 per cent body weight or 15.1 L., for the average individual in this study. The average of the ranges of variation from the third interval (when equilibration had probably been achieved) until the end of the infusion was 29.3 per cent (4.4 L.) of the mean sucrose space. The average of the coefficient of variation [6] over this same period was 8.4 per cent (1.3 L.) of the mean sucrose space. In a single study, the smallest range of variation in sucrose spaces from the third hour until the end of the infusion was 11 per cent of the mean sucrose space (Study No. 88 in Tables I and II, and Figure 2). The largest range of variation between the third hour and the end of the infusion was 47 per cent of the mean sucrose space (Study No. 70, Table I). Some large shifts (3.0 L.) were seen to occur within approximately 60 minutes (Studies No. 34 and 37, Figure 1). On other occasions progressive shifts (3.0 to 5.0 L.) of sucrose space were found to extend over six hour periods (Studies No. 38 and 39, Figure 1). These shifts in sucrose space were not predictable as to direction, magnitude or duration.

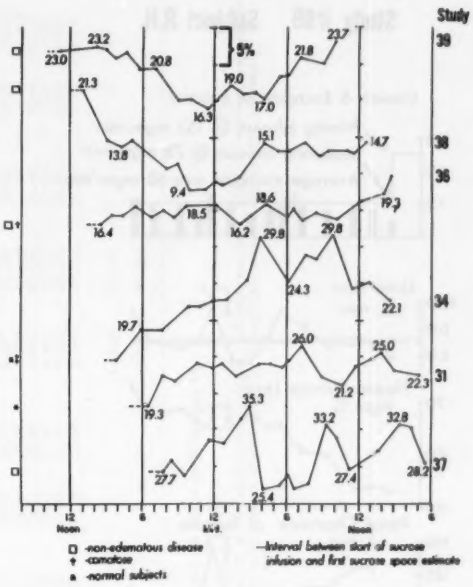

FIG. 1. DIURNAL VARIATIONS IN SUCROSE SPACE
Space in units of per cent body weight.

The experimental model: An examination for random compounding of analytic errors

We sought to determine if the wide variability in sucrose space observed was due to errors or to artifacts in the technique. We investigated this by carrying out several mock experiments. A standard sucrose solution was prepared by weighing the pure sugar and diluting it to a measured

[4] Standard error is used as synonymous with standard deviation.

[5] See Appendix for standard error estimate by Monte Carlo Method.

[6] Coefficient of variation $= \frac{\text{standard deviation} \times 100}{\text{mean}}$.

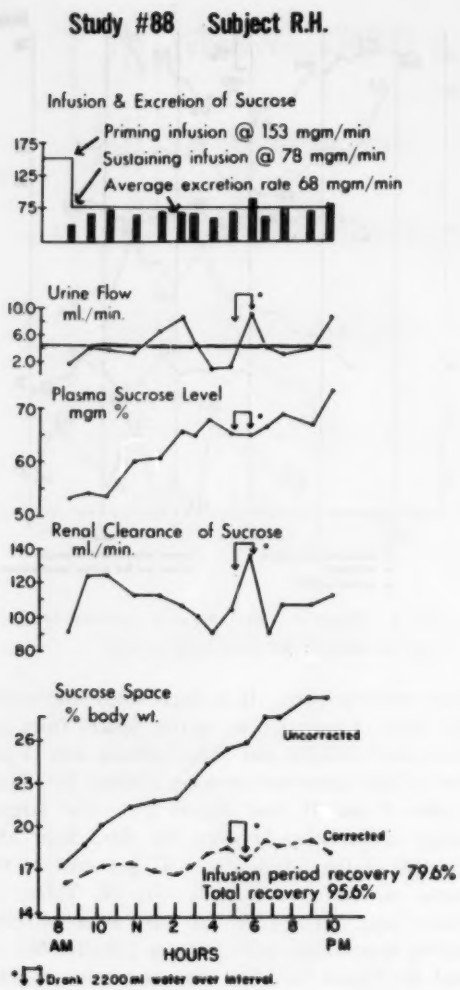

FIG. 2. SUCROSE DISTRIBUTION IN STUDY 88

Although the average sustaining infusion rate of sucrose was 78 mg. per minute, it should be noted, as is shown in Table II, that the rate from 8 a.m. to 4 p.m. was 76 mg. per minute, while that from 4 p.m. to the end of the study was 81 mg. per minute.

volume. This solution was then pumped through the infusion machine for measured intervals of one hour, and the measured volume of each interval was collected in tared volumetric flasks. The volume delivered at each interval was then weighed and compared to the volume that was calculated from the stroke counter. At the end of the experiment, each volumetric flask was brought to volume with distilled water and an aliquot taken for chemical analysis, along with an aliquot of the infusion fluid. The amounts of sucrose delivered at each interval were compared gravimetrically and chemically. Assuming the gravimetric measurement of sucrose as 100 per cent, the chemical recovery of sucrose for each experiment was 99.2, 102.1 and 97.8 per cent, respectively. The difference between the hourly amounts of sucrose determined chemically compared to the same amounts calculated from the stroke counter showed a coefficient of variation of ± 2.58 per cent in one six hour experiment and ± 2.13 per cent and ± 1.44 per cent, respectively, in each of two 10 hour experiments. The coefficient of variation for these three experiments when pooled was ± 1.8 per cent. The total amount of infusion delivered during a patient's study was checked on six occasions, including one 24 hour infusion, by weighing the infusion solution and container at the beginning and end of the infusion period. In these studies, the mean discrepancy between metered infusion delivery and weighed infusion delivery was 1.7 per cent, with a maximum discrepancy of 3.6 per cent.

The possibility that the compounding of analytic errors and pump errors could account for the occurrence of apparent shifts of sucrose space was also explored by using the Monte Carlo Method (see Appendix). Our observed errors of 0.3 per cent for the pump, 2.0 per cent for blood sucrose analysis and 2.0 per cent for urine sucrose analysis were randomized to observe the consequences of these additive errors on successive estimates of sucrose space. The coefficient of variation of a series of "25 intervals" was less than 3.0 per cent of the mean sucrose space. In the unlikely circumstance that all errors (magnitude of one standard error) increased the calculated space at one interval and decreased the space at the next interval, the difference in space would have been 6 per cent of the mean sucrose space. Consequently, the observed variations of EFV in our subjects greatly exceeded those which could be attributed to random compounding of analytic and pump errors.

Sucrose losses

An attempt was made to account for all of the sucrose infused by measuring its recovery in the

TABLE II

The calculation of sucrose space and correction for sucrose losses *

Interval	Time	Infusion†	Plasma sucrose end of int.	Mean plasma sucrose level	Sucrose dose infused	Cumulative dose	Urine sucrose	Cumulative excretion	Dose retained	Uncorrected sucrose space		Corrected urine sucrose§	Corrected cumul. excretion	Corrected dose retained	Corrected sucrose space	
	min.	*mg./min.*	*mg./100 ml.*	*mg./100 ml.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*L.*‡	*% body wt.*	*Gm.*	*Gm.*	*Gm.*	*L.*‡	*% body wt.*
1	87.2	152.6	53.6	53.6	13.309	13.309	4.348	4.348	8.961	15.37	16.71	4.547	4.547	8.762	15.02	16.33
2	67.4	75.2	54.2	53.9	5.069	18.378	4.512	8.860	9.518	16.24	17.65	4.718	9.265	9.113	15.54	16.89
3	49.3	75.3	53.6	53.9	3.711	22.089	3.323	12.183	9.906	16.89	18.36	3.475	12.740	9.349	15.94	17.33
4	88.4	75.6	60.1	56.9	6.684	28.773	5.671	17.854	10.919	17.67	19.21	5.930	18.670	10.103	16.35	17.77
5	72.7	75.7	60.6	60.4	5.506	34.279	4.878	22.732	11.547	17.60	19.13	5.101	23.771	10.508	16.02	17.41
6	67.6	76.6	66.5	63.6	5.179	39.458	4.604	27.336	12.122	17.54	19.07	4.814	28.585	10.873	15.74	17.11
7	47.5	75.9	65.3	65.9	3.604	43.062	3.049	30.385	12.677	17.70	19.24	3.189	31.774	11.288	15.76	17.13
8	44.5	75.9	68.2	66.8	3.380	46.442	2.744	33.129	13.313	18.34	19.94	2.869	34.643	11.799	16.26	17.67
9	64.8	76.4	64.5	66.4	4.952	51.394	4.482	37.611	13.783	19.12	20.77	4.687	39.330	12.064	16.73	18.18
10	59.0	79.5	64.1	64.3	4.693	56.087	5.183	42.794	13.293	19.02	20.67	5.420	44.750	11.337	16.22	17.63
11	58.7	81.3	67.1	65.6	4.774	60.861	3.506	46.300	14.561	20.42	22.20	3.666	48.416	12.445	17.45	18.97
12	51.5	81.4	69.4	68.2	4.194	65.055	3.829	50.129	14.926	20.13	21.88	4.004	52.420	12.635	17.04	18.52
13	84.0	81.8	66.8	68.1	6.868	71.923	6.189	56.318	15.605	21.08	22.91	6.472	58.892	13.031	17.60	19.13
14	63.5	81.0	72.7	69.7	5.141	77.064	5.030	61.348‖	15.716	20.74	22.54	5.260	64.152	12.912	17.03	18.51
0–24 hr. post-infusion urine							11.312	72.660‖				11.829	75.981			
24–48 hr. post-infusion urine							1.039	73.699‖				1.086	77.067			

* These data are from Study No. 88 (a 28 year old normal male weighing 92.0 Kg.).

† Infusion A (4.30 per cent sucrose) was used through Interval 9; infusion B (4.54 per cent sucrose) was used for Intervals 10 through 14.

‡ Correction for plasma water (0.92) was applied at this point. Sucrose space $= \frac{\text{grams retained}}{\text{mean plasma level, Gm. per L.}} (0.92)$.

§ These values were obtained by dividing the uncorrected urine sucrose for each interval by 0.956, the fraction of the total sucrose infused that was excreted. Then the sucrose dose retained within the body at each interval was recalculated for the cumulative results of this correction. Finally, sucrose space was recalculated on the basis of the corrected sucrose dose retained.

‖ Initial recovery = 79.61 per cent; 24 hour recovery = 94.29 per cent; and 48 hour recovery = 95.63 per cent.

TABLE III

Variability of sucrose recovery in repetitive studies (normal subjects)

Subject initials	Study No.	Infusion interval	Sucrose			Recovery during infusion period	Recovery 0–24 hrs. post infusion	Recovery 24–48 hrs. post infusion	Total recovery
			Infused	Average plasma level	Highest plasma level	Total sucrose infused			
		hrs.	*Gm.*	*mg./100 ml.*	*mg./100 ml.*	%	%	%	%
R. H.	73	15	81.9	60.9	71.7	74.6	9.5	0.9	85.0
	78	15	66.6	57.5	63.6	81.3	15.3	0.95	97.6
	88	15	77.0	65.0	72.7	79.6	14.7	1.3	95.6
	111	15	86.0	75.7	90.7	76.8	15.0		91.8
	176	6	34.1	50.8	59.2	55.7	30.3		86.0
R. S.	70	15	97.4	84.0	95.3	71.6	11.8		83.4
	75	6	26.9	52.4	57.1	67.7	29.9		97.6
	77	6	33.3	64.1	69.4	63.1	29.0		92.1
	87	6	42.3	76.9	83.7	63.7	27.9		91.6
	90	6	40.9	58.7	72.6	63.7	30.4		94.1
E. H.	52	6	36.2	50.0	57.7	68.4	23.3		91.7
	59	6	39.5	57.9	71.0	66.2	23.8		90.0
	68	6	42.4	57.7	68.6	70.5	23.5		94.0
	69	6	31.2	43.2	46.5	71.2	22.8		94.0
J. S.	74	6	31.1	51.8	56.8	59.6	22.1		81.7
	79	6	31.5	53.3	60.1	60.6	24.6		85.2
	80	6	11.6	63.2	74.9	67.1	29.2		96.3

urine. In 17 sucrose infusions extending over 10 to 24 hours, the mean urine recovery was 91.4 per cent (S.D. ± 8.5). The completeness of urine sucrose recovery was unrelated to the concentration of sucrose infused or the plasma sucrose level achieved (Table I). In the same individual in different studies (Table III), the degree of sucrose recovery was unrelated to plasma sucrose concentration, indicating that factors other than those measured were most important in determining loss of sucrose. The total sucrose recovered varied as much as 10 per cent in the same person in successive studies (Table III).

The urines were pooled from the infusion period and, in addition, collected for at least the following 24 hours. Twenty-four to 48 hour postinfusion urines were collected after studies on 28 nonedematous individuals. These urines were found to have a mean sucrose content of 2 per cent of the infused dose.[7] Forty-eight to 72 hour postinfusion specimens were collected from three persons, and sucrose in this urine accounted for 0.1 to 0.5 per cent of that infused with a mean of 0.3 per cent. Because of the insignificant amounts of sucrose excreted after 24 hours post infusion, urine was collected routinely for only the first 24 hours post infusion, and this amount was added to the quantity recovered during the infusion period to determine the total sucrose recovery.

In 10 patients with biliary fistulas, six hour sucrose infusion studies were done to learn if sucrose losses in the bile could account for our incomplete recovery. Biliary sucrose could be detected in trace amounts in each study, but the maximum of sucrose loss, with an assumed 24 hour bile flow of 1.5 L. would have been 0.6 per cent of the sucrose infused.

Plasma from two individuals having poor sucrose recovery was incubated for six and eight hours, respectively, in a sucrose solution, and no change in sucrose content was found, either by copper reduction or resorcinol methods. On six occasions, blood specimens were split into two fractions immediately after collection. One was immediately centrifuged and plasma was separated from the red blood cells and blood filtrates prepared immediately; the other specimen was stored in the refrigerator overnight as whole

[7] Six hour infusion studies in 33 edematous individuals had a mean total sucrose recovery of 71.1 per cent (S.D. ± 18.3). The urine collected between the twenty-fourth and forty-eighth hour post infusion of five edematous patients averaged 3.9 per cent of the infused sucrose.

blood. The plasma sucrose level in the whole blood specimen was identical with that of the separated plasma sample in each case.

The possibility was explored that the degree of unexplained sucrose loss was related to the range of sucrose space variations. In Table I, it can be seen that the range in space variation was unrelated to the magnitude of sucrose loss. In five studies where there was a total sucrose recovery of 97 to 103 per cent, there was a range of sucrose space variation (considering only spaces calculated after three hours of infusion) averaging 29.5 per cent of the mean sucrose space. Seven studies with total sucrose recoveries of 91 to 94 per cent showed an average range of 31 per cent of the mean sucrose space for each study. The five other studies had a total sucrose recovery of 83 to 90 per cent, and they had an average range of variation of 26.5 per cent of their respective, mean sucrose space.

Role of fluid intake, urine flow and changes in body weight

During the course of these tests the infused water averaged from 95 to 115 ml. per hour and the total 24 hour infusion volume ranged from 2,300 to 3,300 ml. An additional 300 to 1,000 ml. of oral fluid intake was ingested by six subjects studied for 24 hours. Consequently, the total fluid intake during the six 24 hour infusions varied between 2,300 and 4,000 ml. There was no evidence that sucrose space was increased by intravenous fluids given at this rate or by oral fluids given at a rate not exceeding 250 ml. per hour. However, with greater water loads administered rapidly, larger, but inconsistent, sucrose space changes occurred in the following two instances. In Study No. 55 (Figure 3), a 1,000 ml. water load given over 20 minutes by mouth was associated with a 500 ml. (approximately 0.6 per cent body weight) increase in sucrose space. In Study No. 88 (Figure 2), a 2,200 ml. oral water load given over 30 minutes was associated with a 700 ml. (0.8 per cent body weight) decrease in sucrose space during that period, and was followed by a 1,300 ml. (1.4 per cent body weight) increase in sucrose space in the next hour when 10 mU. of vasopressin was given intravenously.

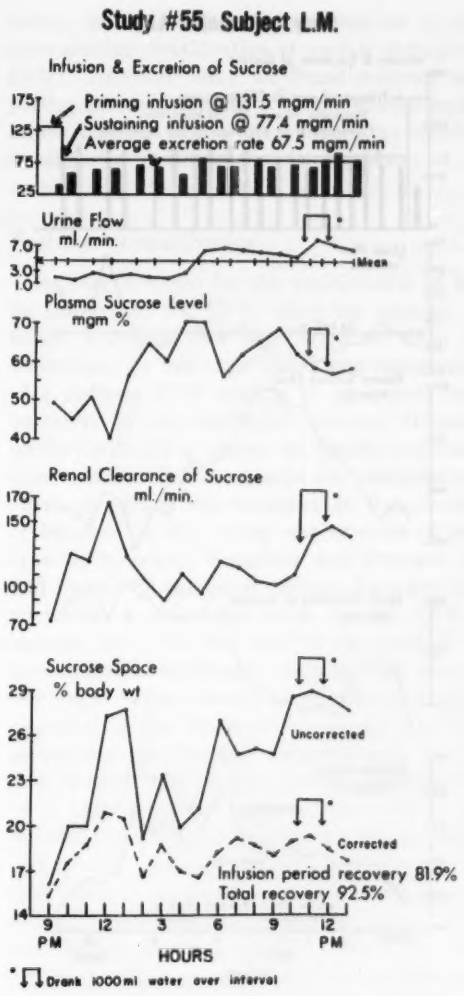

FIG. 3. SUCROSE DISTRIBUTION IN STUDY 55

Urine flow varied widely among individuals and within a single study. In Study No. 34 (Figure 4), the urine flow varied between 0.75 and 1.5 ml. per minute throughout the first 20 hours of infusion with no constant relationship to sucrose space shifts. However, during the last four hours of the same study a sharp increase in urine flow did coincide with a significant decrease in sucrose space. In Study No. 88 (Figure 2), urine flow varied from 0.75 to 9 ml. per minute, changing inversely with the minimal shifts in sucrose space observed. The

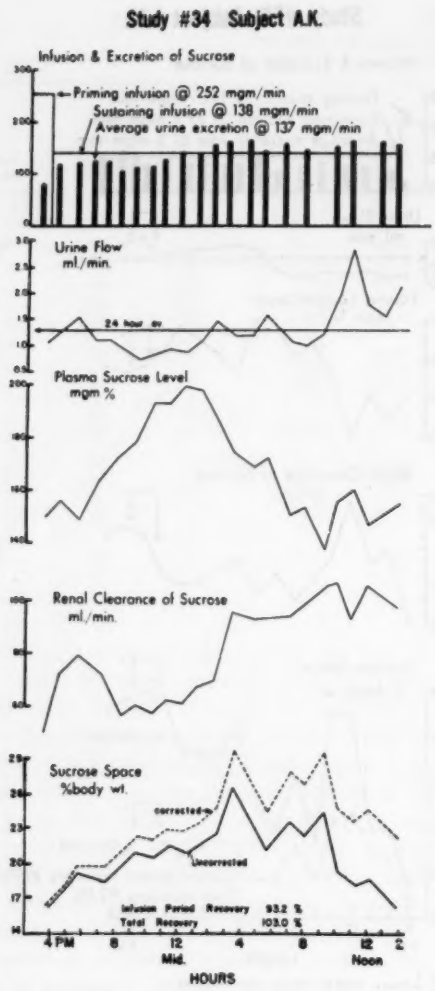

FIG. 4. SUCROSE DISTRIBUTION IN STUDY 34

remaining studies showed lack of a consistent relationship between urine flow and space changes. Changes in urine volume and renal sucrose excretion appeared to account for only a minor portion of the changes in EFV observed.

Changes in body weight were not checked during the six 24 hour sucrose infusions. In four 17 to 18 hour infusions (Studies No. 49, 51, 54 and 55) done in a similar fashion, body weight was determined by having the subject weighed on an in-bed scale at each interval when sucrose space was calculated. During these later four studies, body weight varied from + 0.5 to − 5.5 pounds during the infusion. In the two studies in which weight losses of 4.0 and 5.5 pounds were observed, no significant correlation could be made between changes in body weight and changes in sucrose space.

Role of time of day

These studies were started at various hours of the day. In Figure 1, the possibility was examined that the variations in space represented a diurnal pattern. The studies were arranged in the order of the hour of the day when the infusion was started. No recognizable pattern of space change was correlated with the time of day, or the duration of infusion.

DISCUSSION

The calibrated infusion difference method, like all techniques which estimate extracellular fluid volume, is based on certain premises, the first being that EFV is a discrete volume although made up of subcompartments. The simplest view is of the vascular pool communicating with the major extravascular pool. Actually, the different tissues and organs may vary widely in the relative size of their vascular as well as their extravascular pools, and consequently, estimates of extracellular fluid volume represent sums of these different pools.

Following the injection of an indicator into the vascular compartment, there is an initial rapid vascular mixing phase followed by a slower phase during which the indicator diffuses into the extravascular pools. Since the body tissues vary both in blood volume and perfusion rate, this second extravascular diffusion phase probably differs among organs as to time required for indicator equilibration (12, 13). This major extravascular portion is sampled indirectly through the vascular pool; therefore, to estimate extracellular volume reliably in a short time, rapid diffusion of the indicator must be achieved between the vascular and extravascular pools.

The calibrated infusion difference method as described by Deane, Schreiner and Robertson (7) had four theoretical conditions intended to insure equilibration of the indicator throughout EFV. They felt that: *1*) The rate of indicator infusion

should be constant and accurately measured; *2*) the rate of loss of the indicator should be accurately known from urine analysis; *3*) the rate of diffusion of the indicator should be accurately evaluated from serial measurements of its concentration in the blood and from serially calculated EFV values (a constant blood level was not considered an essential criterion for equilibrium); and *4*) the EFV was assumed to be a pool of constant size. Equilibration of the indicator was thought to be demonstrated when space measurements remained unchanged in successive estimates. In our studies, infusion of the indicator was controlled and renal losses were accurately measured. However, we did not observe a constant EFV and consequently had difficulty evaluating the time at which indicator equilibrium was achieved.

When we began these studies, we prolonged the infusions to explore the possibility that a six hour infusion was not long enough to allow for indicator equilibration throughout EFV. The classic study of Cotlove (14) had suggested that EFV in rats has two distinct compartments: the rapidly equilibrated "functional" EFV plus the slowly equilibrated connective tissue water compartment. Conway (15), in a careful re-examination of Cotlove's data, concluded that the evidence did not support the existence of a slowly equilibrating connective tissue water compartment. Cheek, West and Golden (16), in studies on rats, added further evidence to support this latter view. They found agreement between a six hour inulin space and sodium and chloride spaces after corrections were made for intracellular and skeletal fractions of these ions. For all our studies, when the corrected sucrose space during the fourth interval was compared with the corrected space for the thirteenth interval, a mean decrease of 0.7 per cent body weight was noted. A similar trend was noted in those studies extending to 24 hours. Although this difference is not statistically significant, it certainly is contrary to what should occur with such prolonged infusions, if a significant slowly equilibrating compartment were present.

Even with infusions extending to 24 hours, EFV did not become constant and the plasma sucrose concentration continued to vary significantly, thus precluding the achievement of constant absolute equilibration of sucrose throughout EFV. However, since we found evidence supporting previous reports (7) that sucrose equilibrates rapidly, the inability to achieve absolute equilibration is believed to be the result of the continuously changing EFV.

Problems of equilibration

Indirect evidence for the equilibration of inulin throughout the EFV, using the constant infusion technique, has been based on three observations: *1*) the achievement and maintenance of a constant EFV volume in successive measurements by the calibrated infusion difference method with *2*) a plateau in plasma concentration and/or *3*) a reproducible disappearance curve of plasma concentration at the cessation of infusion (6, 8). Using one or more of these criteria, Schwartz, Schachter and Freinkel (6) and Deane (8) presented evidence for a five hour equilibration period for inulin in the EFV of normal men. On the basis of the ratio of the free diffusion coefficients of inulin and sucrose, one might expect sucrose to equilibrate in about one-third of the inulin time. In the hind limb preparation of the cat, Pappenheimer, Renkin and Borrero (12) report sucrose disappearance at a rate somewhat greater than can be accounted for by its diffusion rate relative to inulin.[8]

The view that such substances as sucrose diffuse slowly into connective tissue was substantiated by White and Rolf (17) in tendon, where there is an absence of capillary network. Skin, however, which contains a large percentage of connective tissue and an adequate blood supply, has been shown to be readily permeable to sucrose inasmuch as sucrose space equilibrated with sodium space in 30 minutes (17).

On the basis of having achieved a plateau in serial EFV values, Deane, Schreiner and Robertson (7) indicated that sucrose had reached equilibration in the body water within two to three hours. In our studies, EFV variations

[8] The Einstein-Stokes diffusion radii (effective molecular radii, cm. $\times 10^8$) of glucose, sucrose and inulin are 3.6, 4.4 and 15.2, respectively. The free diffusion coefficients at 37° C. ($cm.^2$ per sec. $\times 10^{-5}$) are: glucose, 0.90; sucrose, 0.75; and inulin, 0.21 (12).

ranged around a mean sucrose space value which was usually close to that space calculated at two hours. Similarly, the sucrose space estimate made by measuring the final interval blood sucrose and the 24 hour postinfusion urine sucrose was close to the two hour value. Thus, virtual sucrose equilibration was achieved early but, because of changing EFV, we cannot claim to have observed absolute or complete equilibration of sucrose throughout this space.

Extrarenal sucrose losses

The extrarenal sucrose losses mentioned in the Results are another factor to be considered in this sucrose space variability. The reports (18, 19) of "sucrose nephrosis" after massive doses of this sugar in man and dog suggest that part of the sucrose loss might be secondary to intracellular accumulation of this agent in the renal parenchyma. White and Rolf (20) also mentioned the kidney as a major site of intracellular accumulation, since losses were greater in the intact dog than in the nephrectomized dog. Intracellular sequestration in liver and spleen is one type of extrarenal loss which has already been reported in nephrectomized rats by White and Rolf (17). They found no evidence that this was a cyclic phenomenon and concluded from their own data and that of Giebisch, Berger and Pitts (21) from nephrectomized dogs that this intracellular penetration was at a constant rate.[9] Sucrose once within the cell is apparently retained or metabolically degraded. This type of intracellular loss seemed to be the basis for the majority of the extrarenal losses we observed. Moreover, as cited in Results, our studies showed that the magnitude of this extrarenal loss had no relationship to the variability of sucrose space.

Our modifications of the calibrated infusion difference method

Because of the changing level of plasma sucrose and the probable lag in consequent changes in interstitial sucrose, plasma samples at the end of each interval seemed an inappropriate basis for EFV calculation. As a correction for this, we have substituted the mean of the plasma sucrose at the beginning and end of each interval. This change has modulated the extremes of change in EFV previously calculated, but has left intact the major evidence for a continuously varying EFV.

The other important modification was the correction for the constant rate of extrarenal sucrose loss or metabolism. Gaudino and Levitt (9) reported that inulin was quantitatively recovered in the urine within four to six hours following the end of the infusion. Although Schwartz, Schachter and Freinkel (6) found that 95 per cent of the inulin eventually recovered was excreted within the first 12 hours, they did not recover all of the infused inulin even after 36 hours of urine collection. Levy, Ankeney and Berne (22) proposed a correction for this inulin as yet unexcreted which was based on the observation that the logarithmic plot of excretion is linear for the second two and one-half hours of a five hour postinfusion urine collection. Using this linear slope for the excretion rate, they extrapolated to infinity the amount that would eventually be excreted. They made no correction for metabolic loss. Deane and Smith (5) reported that sucrose as well as inulin was apparently lost through metabolism in prolonged infusions but did not propose any correction for this factor in EFV measurements by the constant infusion difference method.

In our studies, extrarenal loss was suggested by the observation that sucrose space increased with time up to the magnitude of 60 per cent body weight. Even with urine collections for 72 hours post infusion, we were frequently unable to recover more than 90 per cent of the infused sucrose. Ikkos (23) reported the finding that sucrose space increased during the course of infusion to an unlikely size, but did not report the degree of sucrose recovery in urine. The inability to recover all of the infused sucrose, if not taken into consideration, resulted frequently in spuriously large estimates of EFV where this space was calculated by the calibrated infusion difference method. This loss of sucrose might be due

[9] This assumption can eventually be tested in studies similar to ours, by using C^{14}-labeled sucrose. This could be done by checking the plasma for evidence that C^{14} was appearing in glucose, fructose or other metabolic fragments at a constant rate. It might also be possible to check the expired CO_2 to determine the degree of variation in specific activity.

to metabolism or to long-term sequestration in some tissue without immediate degradation. We have used a correction factor which is based on the assumption that the total urinary sucrose recovery, including urine of the 24 hours post infusion, divided by the total sucrose infused, described a constant rate of sucrose recovery. An example of a study with the correction applied is illustrated in Table II and Figure 2. This correction, which is based on the constant rate of loss appears to be an empirically valid assumption on the basis of the slope of the uncorrected sucrose space plot. Furthermore, graphs of the successive changes in sucrose space, when corrected over 24 hours, appear to describe variations over a constant mean space. Yet another calculation of sucrose space which does not involve this correction for sucrose metabolism was made by dividing the amount of sucrose recovered in the urine during the 24 hours after the infusion by the plasma sucrose level found at the end of the infusion. This postinfusion recovery space was calculated in the 17 infusions of over 10 hours' duration, and the average difference of this space from the final interval space corrected for recovery was found to be approximately 6 per cent (Table I). This average difference of 6 per cent is less than the standard deviation for successive intervals beyond the third hour corrected for recovery (8.4 per cent). Since the mean of postinfusion recovery spaces was smaller than the mean of final interval corrected spaces, collection of urines for 48 hours post infusion would have increased the postinfusion recovery space and materially decreased the difference between them.

Clinical utility of six hour infusions

The variability in EFV in prolonged infusion studies naturally raised questions concerning the clinical usefulness of sucrose space studies of five to six hours' duration. The mean sucrose space of our 17 prolonged infusion studies was 19.4 per cent body weight. When only the eight normal subjects with 12 studies were considered, the mean was 20.5 per cent body weight. These means are somewhat higher than the 17.6 per cent found for four normal individuals by Deane, Schreiner and Robertson (7). However, in view of the small normal populations being compared and their probable variation in body fat content, these differences are probably not significant.[10] A more important question posed is: How well does an infusion of five to six hours define the EFV as revealed by prolonged infusions? Although the coefficients of variation for sucrose spaces beyond the third hour averaged 8.4 per cent, in 11 of 17 studies the average sucrose spaces calculated for the fourth, fifth and sixth hours agreed within 5 per cent of the mean values for the entire infusion. In only one study did the mean of the fourth, fifth and sixth hours differ more than 15 per cent from the mean of space values for the entire infusion. For this reason, we concluded that a six hour sucrose infusion, using the calibrated infusion difference method, usually described the mean EFV as found by prolonged infusion, but it does not describe the range of EFV variation observed with prolonged infusions. The mean values for the EFV of the first three hours varied more widely from the mean of the entire infusion than did the mean EFV of the fourth, fifth and sixth hours. This would indicate that equilibrium of sucrose was not attained during the first two periods. Nevertheless, the mean of all one to six hour values in a study agrees very well with the mean of an entire prolonged infusion (13 of 17 agree within 5 per cent, but three of 17 differ by more than 15 per cent). Thus, in nonedematous subjects, a six hour sucrose infusion provides a reasonable estimate of EFV, although there is a chance for significant deviation in any one set of observations.

Evidence for EFV change

Our evidence suggests that EFV, as measured by sucrose, changes within the short intervals necessary for space measurement. The reported experience of others using a variety of labels and techniques provides evidence for variability in EFV over short intervals. However, the lack of sufficient control information in normal subjects

[10] We have found, in data from a group of 46 adult males, a meaningful correlation between sucrose space and body fat. The following equation has a negative regression coefficient of 0.441 with a p value < 0.01. Sucrose space (per cent body weight) = 20.1 per cent − 0.173 (per cent fat − 18.7 per cent).

makes the evaluation of these data difficult. Stewart and Rourke (24) reported on the single injection method using a thiocyanate label. Their report gave early evidence of large increases in EFV in postoperative patients having had ether anesthesia. Later evidence (25, 26) using similar techniques reported decreases as well as increases in EFV when ether, as well as other anesthetic agents, was used. The single injection method with sodium thiosulfate as indicator has shown unpredictable changes in EFV in postoperative patients (27). When this same technique was used in normal subjects, some of the results suggested the possibility that EFV could change within the period of measurement (2, 28). Ryan, Pascal, Inoye and Bernstein (29), using single injections of sodium sulfate-S^{35}, found evidence of space increasing and decreasing within the few hours of a study. Recently, Poulos (30) reported on his "constant-change" modification of the single injection technique (using sucrose and inulin) and observed in normal subjects shifts equivalent to 30 per cent of the mean space. Crawford and Gaudino (31), using constant rate infusions of inulin, found unpredictable fluctuations from minus 16 to plus 83 per cent of the initial EFV, following the administration of anesthetic agents or saline infusions. The random nature of these shifts made causal connection with the treatment seem doubtful.

However, any EFV estimate is a function of the indicator and technique used, the body composition and the net effects of physical and biochemical forces acting upon the indicator. This is emphasized here to point out the difficulties that might be encountered in using two or more indicators simultaneously for the purpose of verifying the volume, or changes in volume, of EFV. A promising intercomparison could be made by the use of radioactive indicators, which would permit EFV calculation, both from chemical and from radioassay data of the same indicator. Thus, an important problem raised by these studies is the need for a technique to verify and measure precisely the changes in EFV as reported here.

We believe that the changes observed in sucrose space are due to physiologic shifts in body water which occur from unknown forces. These variable shifts have been observed in previous reports, but have been ignored or ascribed to induced factors and to laboratory and clinical errors (24–27, 30, 31). Since our subjects (except Study No. 88) were not manipulated during the studies and our errors were small, these explanations do not fit our data.

SUMMARY AND CONCLUSIONS

A) A calibrated sucrose infusion technique with extracellular fluid volume (EFV) calculated by the difference method has been described and evaluated in 17 studies of 10 to 24 hours' duration.

B) Indirect evidence is provided that parenterally administered sucrose is metabolized in man. An empirically satisfactory correction for the rate of sucrose metabolism has been applied to the calculation of EFV by the difference method and compared with EFV calculated by the post-infusion recovery method.

C) EFV, as measured by the calibrated infusion difference method, has been observed to exhibit frequent variations of significant magnitude in control subjects, and it is considered reasonable that these measurements describe real fluctuations in EFV.

D) A six hour sucrose infusion usually describes the mean EFV of an individual, but probably does not describe the normal variability of EFV changes for the individual.

E) This variability of EFV demonstrates the complexity of the equilibration problem for any test molecule used in similar techniques and the difficulty in comparing EFV measured by different techniques.

F) There is need for the development of a better technique to verify and measure precisely the changes in EFV as reported here.

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APPENDIX

AN EXAMINATION OF A SEQUENCE OF RANDOMIZED ERRORS BY THE MONTE CARLO METHOD

By OSCAR KEMPTHORNE

The important question that has arisen throughout the present study is whether or not the fluctuations that are observed in the sucrose space of an individual are real fluctuations or are merely artifacts arising from the facts that the ingredients in a computed value are subject to error in the statistical sense, and that the computational procedure incorporates these errors in such a way as to give the appearance of nonrandom fluctuations. An example familiar to students of statistics is the so-called Slutsky effect which may be observed by taking a sequence of random numbers, such as are given in tables of random numbers, and calculating a moving average. It will be found that the resulting sequence of averages exhibits a very marked periodicity and it will generally be accepted that such periodicity is purely an artifact.

It will be seen from the description of the procedure of calculating the sucrose space that the ingredients are the cumulated infused amount less the cumulated excreted amount divided by the blood plasma level, apart from the constant adjustment of incomplete recovery. It is conceivable that a compounding of errors in the measurement of amounts infused and excreted would lead to fluctuations of the magnitude observed. If this were so, the compounding of errors would be a simpler explanation of the observed fluctuations than hypothesizing variations in the true space, defined as the result of the calculation process with observations so replicated as to contain essentially no error.

It is not easy to calculate mathematically the effects of compounding errors (though possibly someone more expert in probability theory could do so) and for such cases a standard procedure is to use the so-called Monte Carlo method (32). This method consists essentially of constructing artificial data subject to the known errors and then observing how a computed value of interest varies. A primary ingredient in such a trial is a sequence of random numbers, which follow the hypothesized random distribution. We assumed that errors of measurement were normally distributed and could have used a table of random normal deviates. Alternatively, as we did, one can take two figure random numbers from a table such as Table XXXIII of Fisher and Yates (33). Converting these to decimals by dividing by 100, we can read from Table IV of Fisher and Yates (or similar tables) the probit corresponding to this proportion, and after subtracting 5 we have a random normal deviate which will have an average value of zero and a standard deviation of unity. Denoting this random number by R, with, for example, an infusion rate of 3.27 Gm. per hour and a relative standard deviation of 0.005 (or 0.5 per cent), we take the amount infused in a particular hour interval to be $3.27\ (1 + 0.005\ R)$. The cumulative amount infused is the sum of the amounts for the successive periods.

The basis of the model used is $V = \frac{\Sigma iv - \Sigma uv}{P_t}$, which assumes EFV to be a single compartment (see Methods). The figures used in this hypothetical case were selected to give the best approximation of the various factors in the volume estimates as observed in our human studies. Σiv, the grams infused, was set at 10.0 for the first interval and 3.27 for all subsequent intervals. The grams infused for each interval was multiplied by the pump error of 0.5 per cent which was randomized as previously described P_t, the blood level in grams per liter, was set at 0.52 with an increment of 0.01 for each interval, multiplied by a randomized standard error of 2.0 per cent. Σuv, the grams excreted for each interval, was set at 3.0 with an increment of 0.01 for each interval, also multiplied by a randomized standard error of 2 per cent. V, calculated with the randomized errors in each factor, was then compared with that V calculated without errors in the same factors. The model was calculated for 25 intervals.

This process was carried out with two complete sets (total, 150) of two figure random numbers. Thus, having each of the three components in the space calculation available in two series with randomized errors, it was possible to calculate for eight cases over 25 intervals, the permutations of randomized errors on sucrose space. The situation was that we have eight sequences as follows:

time	1	2	3	n
"true" space	t_1	t_2	t_3	t_n
"observed" space	0_1	0_1	0_3	0_n

The relative error P_n was calculated as follows: $P_n = 100\frac{0_n}{t_n} - 100$. Then the standard deviation of P_n was calculated, giving the relative compounded error (in this instance, synonymous with coefficient of variation), which ranged from 1.75 to 2.94 for the eight sequences. Using the same representation as above, the largest deviation from zero for the eight sequences ranged from −8.3 to +10.5 per cent. The maximum range of deviation from true space in a series of 25 intervals varied from 7 to 11 per cent. The maximum change over one interval for the eight sequences ranged from 5.1 to 6.1 per cent. Confidence limits were established by constructing a histogram of changes from one time to the next. Over 95 per cent were found to be less than 5.5 per cent and 99 per cent were found to be less than 6.0 per cent.

The coefficient of variation for sucrose space in the 17 patients (313 intervals) was approximately three times the magnitude of the coefficients of variation for space in the eight cases (200 intervals) of randomized errors. Similarly, the range of deviation from mean sucrose space in the 17 patients was approximately three times the similar figure for the cases of randomized error. Only Study No. 88 had sucrose space deviations which approach those of randomized errors, and it had one interval shift that exceeded the 99 per cent confidence interval. Consequently, the deviations in sucrose space found in these patients cannot be attributed just to random analytic and pump errors. Biologic factors must play the major role in the sucrose space deviations described.

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A STUDY OF SUBJECTS WITH ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY: INVESTIGATION OF PLATELET ENZYMES *

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A hereditary abnormality of the erythrocytes was described in Negroes sensitive to primaquine (1). A similar or identical defect has been detected in the erythrocytes of a considerable proportion of non-Ashkenazic Jews susceptible to favism and sensitive to various drugs (2–4). The primary defect of these erythrocytes is probably the markedly decreased activity of glucose-6-phosphate dehydrogenase (5). The cells, however, demonstrate a multitude of secondary abnormalities, namely: a low glutathione (GSH) level; glutathione instability; a decreased glycine incorporation rate into GSH *in vitro;* and an increase in glutathione reductase, aldolase and triphosphopyridine nucleotide (6–9). This hereditary erythrocyte defect is transmitted probably as a sex-linked incompletely dominant trait with various degrees of expressivity of the abnormal gene in affected females (3, 10).

In view of the importance of the hexose monophosphate shunt in glucose metabolism in various tissues and the key role of glucose-6-phosphate dehydrogenase in this metabolic pathway, it is of great interest to determine whether this genetic defect of the erythrocytes is demonstrable in other tissues of the affected subjects. In the present communication we describe the results of an investigation of glucose-6-phosphate dehydrogenase activity in platelets of normal and affected individuals.

MATERIAL AND METHODS

Blood was obtained from: *a*) individuals whose erythrocytes demonstrated a marked glutathione instability by the method of Beutler (11), and no glucose-6-phosphate dehydrogenase activity (most of the subjects were males); *b*) a control group.

Blood was obtained and platelets were isolated by differential centrifugation as described by Gurevitch and Nelken (12). After the third washing the platelets were suspended in 1 ml. saline and were counted by a direct counting technique in duplicate (13). The platelets were then spun down at 1,300 G in an International centrifuge and 0.1 cm.^{3} glass powder was added to the platelet button. The platelets were then triturated for exactly one minute with a glass rod. After one minute 0.5 ml. water was added and grinding was pursued for an additional 10 seconds. Following the trituration the tubes were centrifuged for five minutes at 3,000 G. The supernatant was used for the enzyme activity determinations. All these procedures were performed at 4° C.

Glucose-6-phosphate and 6-phosphogluconic dehydrogenases were assayed by a slight modification of the Kornberg, Horecker and Smyrniotis methods, respectively (14, 15). One-tenth ml. platelet supernatant was added to 2.6 ml. Tris buffer, 0.144 M, pH 7.4, followed by 0.1 ml. triphosphopyridine nucleotide (TPN), 0.001 M, and 0.1 ml. $MgCl_2$, 0.3 M. The mixture was placed in a Beckmann cuvette (light path, 1.0 cm.). One-tenth ml. glucose-6-phosphate, 0.02 M, or 6-phosphogluconate, 0.02 M, respectively, was added to the cuvette. The enzymatic activity was followed by recording the increment in optical density at 340μ at 30 second intervals during three minutes or more. One unit of enzyme activity was defined as an increment in optical density of 0.001 per minute. The results were calculated per 10^9 platelets.

RESULTS

The results are summarized in Table I. The glucose-6-phosphate dehydrogenase level of the platelets in the control group was 127.2 ± 38.5 units per 10^9 platelets, while in the sensitive group the respective mean value was 24.0 ± 17.4 units ($p < 0.001$). On the other hand, no significant difference in the 6-phosphogluconic dehydrogenase activity was detected between the two groups of subjects ($p > 0.7$).

DISCUSSION

Genetically determined enzymatic defects raise a main question of whether the same gene con-

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TABLE I

Glucose-6-phosphate and 6-phosphogluconic dehydrogenase activity in platelets of normal and "sensitive" subjects

Source of platelets	Mean G6PD* activity†	No. of samples	Mean 6PGD‡ activity†	No. of samples
Normal subjects	127.2 ± 38.5	56	75.6 ± 23.7	19
"Sensitive" subjects	24.0 ± 17.4	34	68.5 ± 28.3	15

* Glucose-6-phosphate dehydrogenase.
† In units per 10^9 platelets ± standard deviation.
‡ 6-Phosphogluconic dehydrogenase.

trols a certain enzyme in different tissues. In congenital galactosemia the galactose-1-P uridyl transferase is absent in the red cells and liver (16). Therefore, it is of great interest to determine whether the glucose-6-phosphate dehydrogenase is absent in tissues other than red cells. The present investigation demonstrated that the hereditary deficiency of glucose-6-phosphate dehydrogenase in certain population groups is not restricted to the erythrocytes and that a similar defect is demonstrable in the platelets as well. To exclude the possibility of erroneous results due to differences in platelet fragility, Dr. Nelken was kind enough to perform a platelet fragility test on enzyme deficient platelets and found it to be normal (17). In a number of experiments pyrophosphatase activity was determined and the activity of this enzyme was found to be similar to that found in normal platelets (18). Finally, the finding of similar levels of 6-phosphogluconic dehydrogenase in normal and deficient platelets would strengthen our results. On the other hand, Marks, Gross and Hurwits have found glucose-6-phosphate dehydrogenase activity to be normal in the leukocytes and slightly decreased in the livers of affected individuals (19). There is no explanation at present for the similarity of the findings in erythrocytes and platelets and their difference from the findings in livers and leukocytes.

Glucose-6-phosphate dehydrogenase activity declines in the erythrocyte as a function of cell age (20). Even in deficient erythrocytes the younger red cell population has a higher enzyme level than the older cells (21). The glucose-6-phosphate dehydrogenase in erythrocytes affected by the hereditary defect is more heat labile than in normal cells (4, 22). Therefore, there is a possibility that the enzymatic defect in the erythrocytes is not due to a lack of this enzyme in the cells, but to an enzyme that is qualitatively abnormal and is degraded more rapidly. If this assumption is correct it is possible that erythrocytes and platelets that are non-nucleated cells and presumably unable to synthesize proteins could not compensate for an increased enzyme turnover, while the liver and leukocytes do have such a synthetic potential and therefore could do so. This is our working hypothesis at present. It is also possible that only cells exhibiting a decline in glucose-6-phosphate dehydrogenase as a function of their age will demonstrate this enzymatic defect.

The hereditary defective erythrocytes respond with a hemolysis *in vivo* to the administration of various drugs, such a primaquine, furadantine, para-aminosalicylic acid, sulfanilamide and sulfapyridine (1, 2). No thrombocytopenia, however, was observed by us during a hemolytic event, nor has it been described in the literature. Beutler, Robson and Buttenwieser have shown that there is a relation between the drop in GSH and the appearance of hemolysis *in vivo* (6). They have also shown that GSH of sensitive erythrocytes is destroyed when oxygenated cells are incubated with acetylphenylhydrazine. Furthermore, hemoglobin solutions prepared from red cells which have been incubated with acetylphenylhydrazine were shown to destroy GSH. The recent finding of an enzyme, GSH peroxidase, in erythrocytes that could mediate the oxydative destruction of GSH by a hemoglobin peroxide compound is of interest (23). All these data demonstrate a possible relation between hemoglobin, GSH destruction and hemolysis.

The lack of hemoglobin in platelets might be related to their immunity to the damaging effect of drugs. On the other hand, this could be re-

lated to the short survival time of platelets in general and the great capacity of the marrow to compensate for the peripheral destruction (24). The possible presence of other TPNH generating systems in platelets should also be considered.

SUMMARY

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activity were determined in platelets of individuals whose erythrocytes showed a deficiency of the former enzyme. A significantly lower level of glucose-6-phosphate dehydrogenase was found in these platelets, whereas the 6-phosphogluconic dehydrogenase activity was normal.

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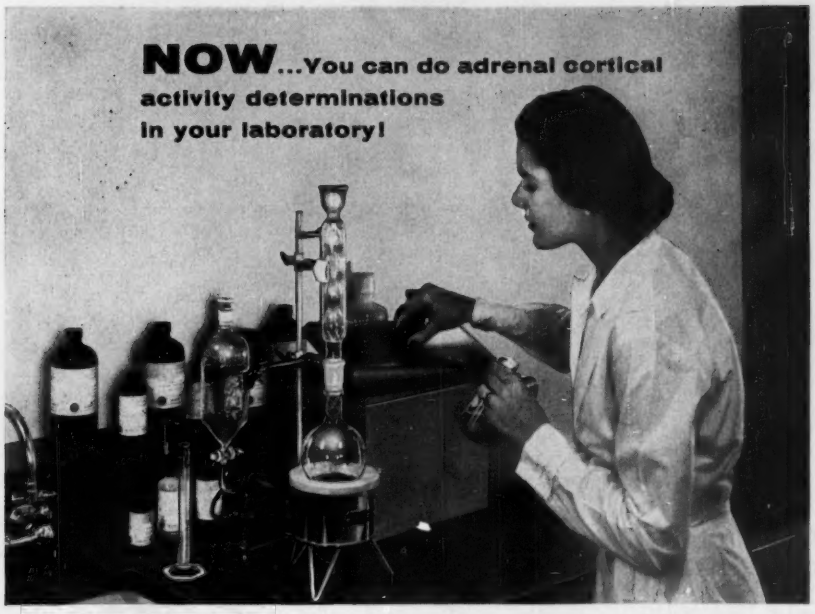

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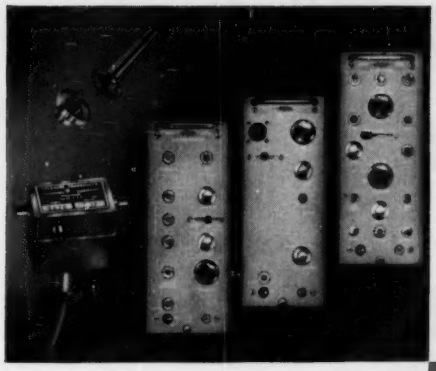

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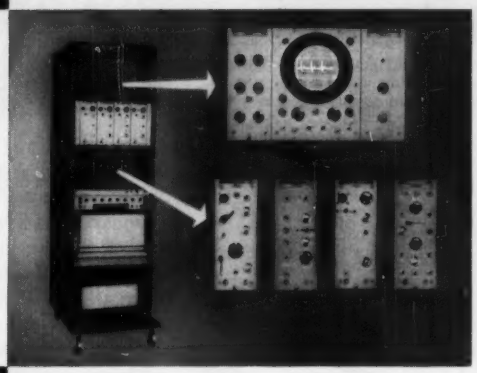

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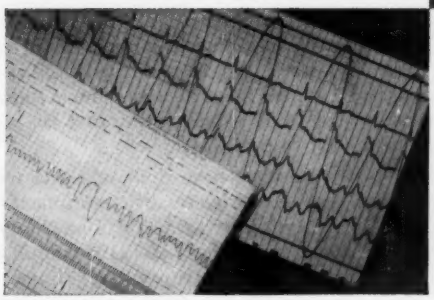

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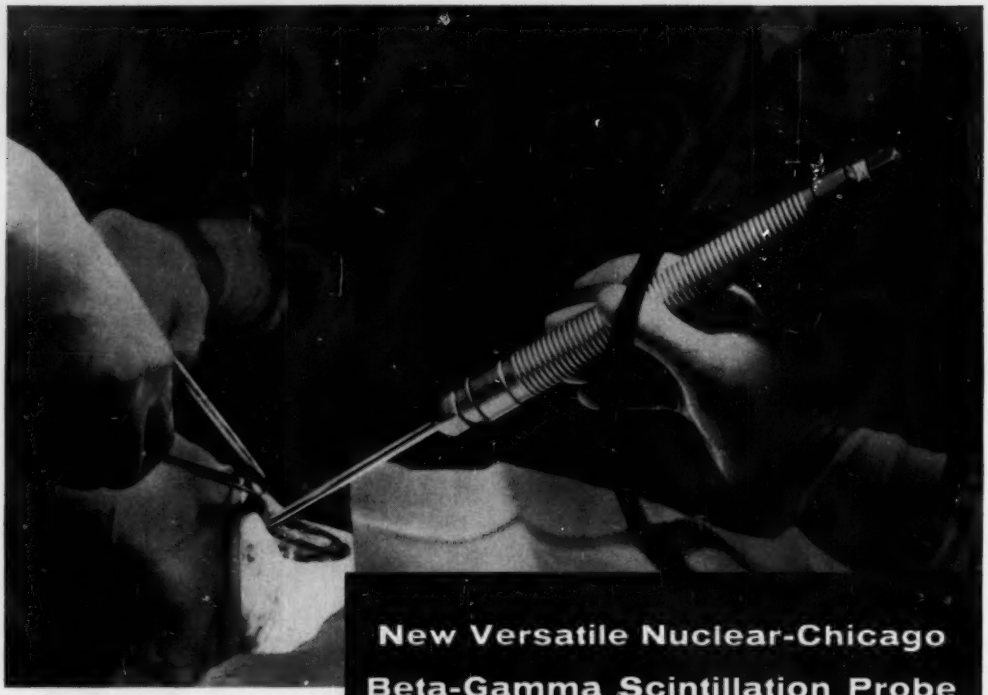

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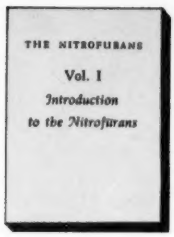

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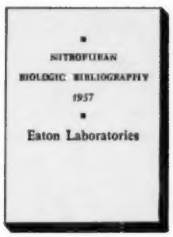

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